11-1985

Plasma Carnitine of the Premature Infant

Rebecca Bryan Smith
University of Tennessee, Knoxville

To the Graduate Council:

I am submitting herewith a thesis written by Rebecca Bryan Smith entitled "Plasma Carnitine of the Premature Infant." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

Dileep S. Sachan, Major Professor

We have read this thesis and recommend its acceptance:

John T. Smith, Michael H. Sims, Vichien Lorch

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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V.L. Cody

Accepted for the Council:

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Vice Provost
and Dean of The Graduate School

PLASMA CARNITINE OF THE PREMATURE INFANT

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Rebecca Bryan Smith

December 1985

ACKNOWLEDGMENTS

An expression of thanks and gratitude are extended to the indivduals involved in this research project as well as all of the academic guidance which enabled me to obtain my Master's degree. Special gratitute is given to:

Dr. Dileep Sachan, my major professor, for the guidance and encouragement given to me while working towards my degree;

Dr. John Smith, Dr. Michael Sims, and Dr. Vichien Lorch, all members of my Master's committee, for their guidance and critique of my thesis, and for their academic teachings which allowed me to complete this manuscript;

Joan Plattsmeir, Dr. Neil Feld, and Dr. Linda Hoersten, of the University of Tennessee Memorial Research Center and Hospital, for their hard work and thought provoking ideas which allowed this research project to be completed;

Pat Pittman and her staff of the Pediatric Laboratory at the University of Tennessee Memorial Research Center and Hospital, for their work obtaining the blood samples used in this study;

Wanda Dodson and Bob Berger, for the laboratory guidance and assistance throughout my graduate studies, and to Andrea Richmond and Karen Bledsoe, as well as all my fellow co-workers, for their contributions which allowed to completion of this research project;

The Department of Nutrition and Food Science for the excellent facilities and support given to me during my graduate work at The University of Tennessee.

ABSTRACT

Premature infants have been shown to exhibit fat intolerance with the administration of exogenous lipid, especially intravenous lipid. Carnitine is required for the efficient oxidation of long-chain fatty acids. Since infants have been reported to be unable to synthesize carnitine, the carnitine and lipid status was investigated in a series of premature infants throughout the entire period of hospitalization. At birth, the plasma carnitine of three groups of appropriate for gestational (AGA) premature infants (n=36), was compared with full term infants (n=10), and with maternal and umbilical cord plasma carnitine levels. The plasma concentrations of carnitine, triglyceride, and in some cases free fatty acids, were determined on each of the premature infants on days 0-5, 7, and then weekly until discharge. The plasma concentration of carnitine and lipid were also determined prior to, and after, the initiation of various types of infant feedings. The feedings consisted of both carnitine-free and carnitine-containing formula/infusates. The relationship of the levels of carnitine and lipid were analyzed between groups to determine if lipid intolerance was associated with a depressed carnitine concentration. The plasma levels of carnitine and lipid were also analyzed to determine their relationship with carnitine, fat, and in the case of triglyceride, with calorie intake.

Results showed that infant plasma carnitine levels at birth were directly related to the maternal plasma carnitine concentration. The infant plasma carnitine level was higher in the younger infants when

compared with the older prematures and full term infants. All groups exhibited a decrease of the ratio of non-esterified carnitine to acid-soluble acylcarnitines soon after birth, which indicated metabolism of lipid by B-oxidation. Most of the infants required an exogenous source of carnitine for the maintenance of the plasma carnitine level. However two infants showed a slight elevation of plasma carnitine while on a carnitine-free diet. This may have been due to either an active biosynthesis of carnitine, a release of carnitine from red blood cells, or a release of tissue carnitine.

All infants responded to dietary carnitine intake with an elevation of the plasma carnitine level. The plasma triglyceride level was directly related to calorie intake, but correlated to fat intake only in the the older premature infants.

A significant negative relationship between the plasma levels of carnitine and triglyceride did not exist in any group. However at the time of peak depression of plasma carnitine, the intake of intravenous lipid and long-chain fatty acids was relatively low. During this time most infants received medium-chain triglycerides as the primary source of exogenous fat, which might explain this unexpected result. Therefore it appears that premature infants tolerate intravenous lipid doses of at least 1.0 - 1.5 g/kg/day and diets which contain a high percentage of total fat from medium-chain triglycerides without evidence of fat intolerance even when they are maintained on a carnitine-free diet.

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CHAPTER I

INTRODUCTION

The survival rate among low birthweight (LBW) infants is increasing due to advances in medical understanding and technology. Information regarding nutritional requirements and metabolism of oxidative substrates in the LBW is limited and vague. Requirements for energy and most nutrients are greater than those for term infants, since most nutrients are stored during the last trimester of pregnancy. However, due to the relative immaturity of metabolic systems, the LBW infant has a limited capacity to metabolize substrates to meet their higher needs for energy.

Many LBW infants are fed parenterally for the first few weeks of life. Lipid emulsions are commonly used as a source of essential fatty acid(s) and calories during the provision of parenteral nutrition. Fat is the most calorie-dense substrate, supplying 9.1 kcal/g. Unfortunately the premature infant does not appear to tolerate moderately large lipid loads. Information regarding the exact mechanism for lipid intolerance is unknown at this time.

Carnitine is an essential biocatalyst for the translocation of long chain fatty acids into the mitochondria for β-oxidation. Biosynthesis of carnitine in the neonate is thought to be limited due to a possible deficiency of γ-butyrobetaine hydroxylase, the last enzyme in the carnitine biosynthetic pathway. Since carnitine is required for the translocation of long-chain fatty acids (LCFA), a relative carnitine deficiency may severely impair the release of

cellular energy. If carnitine is not present in sufficient amounts, this might possibly lead to (1) inability of the infant to gain adequate weight, (2) develop hyperlipidemia along with its associated toxic effects, and (3) it would ultimately affect the length of hospitalization for the LBW infant. Several investigators have studied the relationship between plasma carnitine and plama lipid levels. However, long term serial data is not available concerning plasma carnitine and lipid levels during an entire hospital stay with various nutritional interventions.

CHAPTER II

REVIEW OF LITERATURE

A. Neonatal Metabolism

In the first three hours of extrauterine life, 90% of the infant's glycogen stores are depleted (1). During this time the infant relies increasingly on fat as a metabolic fuel. The levels of glucagon (2), epinephrine and cortisol are increased soon after birth which induce lipolysis in adipose tissue (3). Non-esterified fatty acids (NEFA) are liberated and oxidized to acetyl CoA which enters the tricarboxylic acid cycle or is used for synthesis of ketone bodies. Both of these pathways are useful for sparing blood glucose and provide energy for adequate tissue function.

Fat continues to be important energy source during infancy. Premature infants have a higher caloric requirement due to the lower digestable energy coefficient and higher fecal energy loss (4), and higher energy needs for new tissue synthesis compared with term infants (5). Therefore exogenous fat from formula or parenteral lipid emulsion would appear to be a practical means of providing calories to the LBW infant. Unfortunately, LBW infants do not tolerate lipid well, resulting in an elevation of plasma triglyceride (TG) and/or NEFA. The exact cause of fat intolerance in the LBW infant is unknown.

B. Review of Lipid Metabolism

Plasma triglycerides may be derived from dietary TG or from formation of hepatic very low density lipoprotein (VLDL). Dietary fat consists primarily of TG containing long chain fatty acids, although some infant formulas are supplemented with medium chain triglycerides (MCT) or coconut oil. Long chain triglycerides (LCT) are hydrolyzed in the intestinal lumen to monoglyceride and NEFA prior to absorption. In the intestinal mucosa, TG are resynthesized and released into the lymphatic system as chylomicrons and later enter the venous system via the thoracic duct. During movement from lymph to blood, chylomicrons aquire apoproteins C and E (Apo-C and Apo-E). The Apo-C II activates endothelial-bound lipoprotein lipase (LPL), catalyzing the hydrolysis of TG to NEFA and glycerol. The remaining chylomicron remnant reacts with the Apo-E and is taken up by endocytosis into hepatic parenchymal cells.

Medium-chain triglycerides (MCT) are another source of dietary fat. Some infant formulas have had a portion of the LCT removed and replaced with MCT or coconut oil (59% of the fatty acids are ≤C:12). The MCT are primarily hydrolyzed in the stomach and are absorbed directly into the portal blood system. The MCT do not require emulsification with bile salts, they are not re-esterified in the liver, and they do not accumulate in the plasma.

Plasma TG from endogenously circulating NEFA are synthesized in the liver as very low density lipoproteins (VLDL), which is comprised of primarily TG and Apo-B, E, and C. After VLDL release from the liver,

the VLDL aquires Apo-C II which again activates endothelial-bound LPL causing loss of TG and leading to the release of NEFA and glycerol. The VLDL remnant may then be modified into low density lipoproteins (LDL) or after reacting with Apo-E, it may coat, or be engulfed by phagocytes.

Long chain NEFA which are released from TG or adipose tissue are transported in the systemic circulation bound to albumin. The albumin-bound NEFA are carried to tissues, where they are taken up into subcellular organelles for oxidation. The primary site of β-oxidation is the mitochondria where complete oxidation to acetyl CoA (or to propionyl CoA and acetyl CoA in odd-chain fatty acid oxidation) is accomplished.

Non-esterified fatty acids undergo chain-shortening in the mitochondrial matrix which is surrounded by a double membrane. The inner mitochondrial membrane is impermeable to fatty acyl-CoA. To penetrate, carnitine facilitates intramitochondrial translocation of the fatty acid moeity of an acyl-CoA with the help of carnitine acyltransferase A (CAT A), which is located on the outside of the inner mitochondrial membrane:

Fatty acyl-CoA + Carnitine ---→ Fatty acylcarnitine + CoA-SH

Once inside the membrane, the fatty acyl-CoA is reformed and carnitine is released within the mitochondria, a reaction catalyzed by carnitine acyltransferase B (CAT B), located on the inside of the inner mitochondrial membrane. The activated fatty acid is now available for

chain-shortening by the enzymes of β-oxidation.

Unlike long-chain fatty acids, the medium-chain fatty acids are not dependent on carnitine for transport into the mitochondrial for their subsequent oxidation.

Peroxisomes also oxidize fatty acids by a β-oxidation process. Although carnitine palmitoyltransferase is absent in peroxisomes (6), Kondrup and Lazarow have shown that 17-48% of [1-^{14}C]palmitate is oxidized, or partially oxidized in peroxisomes of rat hepatocytes (7). Both the amount and type of dietary fat can affect the rate of hepatic peroxisomal and myocardial peroxisomal β-oxidation activity. Unlike mitochondrial-produced acetyl CoA, the peroxisomal acetyl CoA is proposed to be committed only to the synthesis of cholesterol. The importance of peroxisomal β-oxidation in the neonate is unknown.

C. Fat Intolerance in the Low Birthweight Infant

Fat intolerance in the LBW infant has been hypothesized to occur from decreased oxidation, decreased tissue uptake of NEFA, or from decreased plasma clearance of circulating TG (either as chylomicrons or VLDL).

Recently, attention has been focused on fat intolerance secondary to administration of intravenous lipid emulsion. Commercial fat emulsions for use in infants are composed of long-chain triglycerides (10% w/v), egg yolk phospholipid (as an emulsifier), and glycerol (to obtain an isotonic emulsion). The fat emulsion particles are metabolized similarly to naturally-occuring chylomicrons (8). The fat emulsion is suitable for peripheral or central infusion, and it may be

administered as a bolus (slowly) or continuously throughout a 24 hour period.

Fat intolerance as a result of intravenous lipid administration may occur at various fat doses (9,10,11,12). Griffin (9) found that when either bolus or continuous infusion of fat emulsion was administered, plasma TG levels would peak and plateau within 5 hours after the initiation of the fat emulsion. The TG plateau was maintained throughout the period of infusion, however there were wide day to day variations in the plateau levels acheived in the individual infants. These variations could not be explained by either mode of infusion or lipid dose.

Hilliard et al. (10) found that AGA premature infants (26-32 weeks at birth) had increased plasma concentrations of TG, NEFA, total lipid, and glycerol when at least 2 g/kg/day or more of lipid was given by continuous infusion. There was no further significant increase in plasma lipids until more than 4 g/kg/day was administered. In the same study, NEFA concentration remained within baseline, pre-infusion range (0.41 ± 0.046 mmol/dl) with infusions of up to 2 g/kg/day of lipid. Infusion of 3-4 g/kg/day of lipid produced very wide variations in NEFA concentration, with mean levels significantly greater than baseline (1.2 mmol/dl) at 96 hours, but the NEFA level was not significantly greater than when the lipid dose was 2 gm/kg/day.

Hilliard found that plasma TG levels were significant above pre-infusion levels (but not in an abnormal range) when a lipid dose of 1 g/kg/day was administered. Preinfusion TG concentration averaged 0.30 ± 0.031 mmol/dl and at 4 hours after the start of lipid infusion,

plasma TG were significantly higher than baseline levels when the lipid dose was 1 g/kg/day. Unlike the results of Griffin, a TG plateau was not maintained throughout the period of lipid infusion. At 8 and 12 hours after the start of lipid infusion, TG had decreased to the upper statistical limit of pre-infusion levels. As the dose was increased above 1 g/kg/day on a daily basis to the maximun dose of 4 g/kg/day, only one 23 week infant obtained a TG level of 2.5 mmol/dl. Thus, it appears that most infants can be given up to 4 g/kg/day of intravenous lipid without evidence of hypertriglyceridemia as long as the lipid is administered as a continous, rather than bolus, infusion.

Cooke et al. (11) recently investigated the lipemic effects of giving bolus infusions of safflower oil-based lipid emulsion (Liposyn, Abbott Labs). Two similar groups of preterm infants (28-34 weeks) were infused with lipid, with doses either 0.34 g/kg/day or 0.68 g/kg/day for 5 days. There was a higher indicence of hypertriglyceridemia (defined as >200 mg/dl) in the group receiving the higher lipid dose (9 of 16 versus 2 of 8 in the 0.34 g/kg/day group). However there was no difference in NEFA levels between the groups despite the difference in lipid dose. Cooke noted that in infants ≤32 weeks, the post-infusion NEFA were greater (NEFA uptake was less) than infants 32-34 weeks gestation.

Andrew et al. (12) observed that although lipid tolerance was related to gestational age, it was also related to weight for gestational age. These infants were given bolus infusions of soybean oil-based lipid emulsion (Intralipid), at a dose of 1 g/kg/day over a 4

hour period. Infants ≤33 weeks gestation and the small for gestational age (SGA) infants of 32-40 weeks gestation had significantly higher concentrations of TG and NEFA during the infusion period and they cleared the lipid more slowly than the AGA infants >33 weeks gestation.

These studies suggest both impaired hydrolysis of the infused lipid and inefficient uptake and/or utilization of lipid in the premature infant. They also suggest that intravenous fat is better tolerated when administered as a continuous infusion.

D. Complications of Lipid Intolerance

The infusion of lipid emulsions have been associated with complications in LBW, premature infants. Hypertriglyceridemia due to lipid infusion has been reported to be associated with hypoxia (13) and exacerbation of existing ventilation/perfusion inequalities (14) which are frequent in premature infants. Lipid emulsion administration has been proposed to inhibit neutrophil function (15,16), and compromise immunity against bacterial infection (17). Therefore caution is taken to avoid large lipid doses, especially in the premature infant.

An increase of plasma NEFA has also been associated with adverse effects. Plasma NEFA in excess of a NEFA to albumin molar ratio of 6:1 can competitively displace bilirubin from its primary binding site (18). This would increase the level of free bilirubin in the blood of infants who are already at an increased risk of developing kernicterus. Kao et al. (19) compared the NEFA:albumin molar ratio of infants of either 28-32 weeks or >32 weeks of age during either a continuous or bolus

lipid infusion. With continuous infusion in infants ≤32 weeks gestational age, the NEFA/albumin molar ratio remained constant only when the lipid dose was <1 g/kg/day. The molar ratio exceeded 10:1 as the lipid dose approached 2 g/kg/day. At a lipid dose of 2.5-3.0 g/kg/day, the ratio increased to 10-14:1. In infants >32 weeks the molar ratio did not approach 6:1 until administration of approximately 2.5-3.0 g/kg/day of lipid. The older infants never attained the higher NEFA:albumin molar ratios (10-14:1) of the infants ≤32 weeks of age. All infants tolerated the intermittent regimes (daily lipid administration for 8 hours) less than when the intravenous lipid was given continuously. With intermittent lipid infusion, the peak ratio was >6:1 in all infants ≤32 weeks at any lipid dose, and they acheived a ratio of 24:1 at lipid intakes of 2.5 g/kg/day. In infants >32 weeks, lipid doses up to 2 g/kg/day did not adversely affect the NEFA:albumin ratio. However, when the lipid dose was increased to 5 gm/kg/day, the molar ratio exceeded 12:1. As with the continuous administration of lipid, infants >32 weeks never attained the molar ratio seen in the more immature infants.

E. Etiology of Lipid Intolerance

It would appear that fat intolerance in the LBW infant is multifactorial and the proposed etiologies are quite numerous. Plasma NEFA may be elevated by the presence of stress-related hormones, a compromised nutritional state (a high glucagon to insulin ratio), or during administration of drugs such as theophylline and dopamine (20) which may initiate and/or potentiate lipolysis in the newborn.

Cellular uptake of NEFA may be poor in premature infants due to relatively low numbers of adipocytes (21). An inadequate source of α-glycerol phosphate (22) due to depleted glycogen stores may prevent NEFA re-esterification with subsequent NEFA accumulation in the blood. Depressed plasma carnitine has also been correlated with elevated levels of plasma NEFA (23,24) during the parenteral infusion of fat emulsion.

Hypertriglyceridemia may result from decreased activity of certain enzyme systems or from the accumulation of endogenously produced or exogenously provided lipid or carbohydrate. Lipoprotein lipase (LPL) activity is age-dependent; infants < 27 weeks have one-third of the reserves of LPL of term infants (25). The LPL activity parallels calorie intake (26), having a very low activity when intake is low and increasing accordingly (27). In animal studies, both viral and bacterial infections have been shown to depress LPL activity (28), leading to elevated plasma triglyceride. Glucose provided to prematures in excess of what can be directly oxidized for energy, 7 mg/kg/min (29), must first be converted into lipid before oxidized for energy. Lecithin-cholesterol acyltransferase was recently found to be deficient in premature infants (30), a condition which could indirectly elevate plasma triglyceride concentration. Cooke et al. (31) reported that hypertriglyceridemia is more common in infants receiving a safflower oil-based lipid emulsion (Liposyn), when compared to a similar group of infants being infused with a soybean oil-based emulsion (Intralipid).

On the other hand, administration of MCT preparations have been

shown to have beneficial results in the neonatal rat (32,33). The medium-chain fatty acids have been shown to support ketogenesis and prevent hypoglycemia in the newborn rat. These effects are attributed to the faster rate of hydrolysis and oxidation of MCT compared to long-chain fatty acids. Unfortunately, there are currently no MCT preparations suitable for intravenous use.

F. Carnitine Metabolism

As mentioned previously carnitine (CNE), 3-hydroxy-4-N-trimethyl-aminobutyric acid, is absolutely required for the intramitochondrial translocation of long chain fatty acids. Lack of carnitine may lead to a relative inability of tissues to oxidize lipids for energy.

Carnitine may also be linked to energy release through other less elucidated pathways. Carnitine has been proposed to be a stimulator of lipolysis in subcutaneous adipose tissue and a recoupler of oxidative phosphorylation in brown adipose tissue of newborns (34). Carnitine may play a facilitative role to stimulate the decarboxylation of branched-chain amino acids (primarily leucine) in skeletal muscle tissue. This is accomplished by increasing the rate of conversion of the branched chain ketoanalogs into carnitine esters for entry into the mitochondria (35). The CoA-SH pool is regenerated, allowing the branched-chain ketoacid dehydrogenase reaction to shift to the right and stimulate further branched-chain amino acid oxidation in order to support gluconeogensis. Carnitine may also function as a transport vehicle for acetyl and medium-chain acyl residues out of peroxisomes, or for transport of the products of peroxisomal β-oxidation to the

mitochondria or the endoplasmic reticulum (6).

Carnitine availability in the mitochondria could theoretically regulate the oxidative metabolism of carbohydrate, glucogenic amino acids as well as lipid (6). By converting acyl-CoA's into acylcarnitines, there is regeneration of the CoA-SH pool in the mitochondrial matrix. Coenzyme-A is then available for the production of acetyl CoA (from pyruvate), succinyl CoA (from α-ketogluterate), branched chain acyl CoA (from branched-chain keto acids) and long chain fatty acyl CoA (from long chain NEFA). All of these are important for release of metabolic energy.

G. Sources of Carnitine

Carnitine may be obtained from endogenous biosynthesis, dietary intake, and in the newborn, from placental transfer. Carnitine is synthesized from protein-bound lysine, methionine, and requires the cofactors ascorbic acid, niacin, pyridoxine and ferrous iron (37). In humans, γ-butyrobetaine hydroxylase, the limiting enzyme in the conversion of γ-butyrobetaine to carnitine was found to be only 12% of adult levels in infancy (37). Decreased activity of this enzyme may indicate that carnitine is an essential nutrient during infancy.

Cofactors necessary for carnitine biosynthesis may be present in deficient amounts in the LBW and may contribute to the infants inability to synthesis carnitine. Cord levels of pyridoxine are lower in premature infants than in term infants (38). Infants on total parenteral nutrition have deficient plasma levels of pyridoxine (39). Rats given the B-6 antagonist, 1-amino-D-proline, show a 60-80%

depression of carnitine production from trimethyllysine (40). Also, LBW infants have reduced iron stores at birth (41). It was shown that iron depleted rat pups had 42% lower liver carnitine levels and an 8-fold increase in triglyceride concentration, even when adequate lysine and methionine were provided in the diet (42). Premature infants deficient in any of these precursors or cofactors may have reduced concentrations of carnitine.

Dietary sources of carnitine include meat and milk products. Milk-based commercial infant formula and human breastmilk are good sources of carnitine. Human breastmilk averaged 75 nmol/ml when measured by Ohtani (43). Commercial milk-based infant formulas contained significant, but variable amounts of carnitine. Commercial infant formula based on soy, casein and egg white proteins contain only minute quantities of carnitine (44). Intravenous amino acid solutions and lipid emulsions are virtually carnitine-free, <1 nmol/ml (45).

In the fetus, carnitine is obtained by placental transfer from the maternal blood supply. Placental transfer of carnitine has recently been elucidated in humans. Using an in vitro, double circuit perfusion design, Schmidt-Sommerfeld et al. (46), concluded that (1) placental carnitine transfer occurs by passive diffusion through extracellular channels, (2) there is stereospecific cellular uptake for the L-isomer, although both the D- and L-isomers diffuse into the placenta similarly, and (3) the human placenta is actively involved in carnitine metabolism, having the ability to esterify free carnitine. The authors also concluded that, based on their calculated maximal transfer of 500 µmol/day and a 13 µmol/day carnitine accumulation during the last

trimester into the fetus (47), carnitine delivery to the fetal circulation is in excess of fetal requirements and fetal biosynthesis would probably not be necessary.

H. Carnitine Acyltransferases

Carnitine is the substrate for various carnitine acyltransferases. These enzymes are essential for acylcarnitine transport through various membranes. Of special importance in lipid oxidation is carnitine palmitoyltransferase (CPT). This enzyme is responsible for activated palmitic acid entry into the mitochondria. The CPT activity is exclusively located in the mitochondria of liver, heart, kidney and skeletal muscle (48). In term newborn infants, there is a significant increase of CPT activity in white adipose tissue mitochondria of term infants 20 hours old when compared with the CPT activity at 0-10 hours of age in the same infants. The CPT activity was lower in prematures when compared with the term infants during the entire 20 hours (49). In newborn rats, CPT levels are low but increase with time. Adult levels of CPT are obtained in liver by 4 days, but not in skeletal muscle until 15 days of life (44). Earlier activity of hepatic CPT probably reflects the importance of ketogensis in the newborn.

Carnitine palmitoyltransferase is proposed to be inhibited by malonyl CoA (51) and intramitochondrial myocardial pyruvate (52), and activated by palmitoyl CoA (53).

The short and meduim chain acyltransferases, substrates mentioned previously, are located in mitochondria, peroxisomes and endoplasmic

reticulum. Their primary function is thought to be protection of the CoA-SH pool and for membrane transport function (6).

I. Plasma Carnitine

1. Factors Affecting Plasma Carnitine Concentration

The plasma carnitine concentration in humans varies with age, sex, endocrine and nutritional status, and disease. The plasma carnitine level required for normal metabolism is not known, since the relationship of tissue and plasma carnitine levels may not be correlated (54). Normal, healthy men have been reported to have plasma values of 57.3 ± 12.8 nmol/ml (mean ± 1 SD), and non-pregnant fertile women of 46.5 ± 12.4 nmol/ml (55). It has been shown that rats injected with androgens have an increased plasma carnitine level, and rats injected with estrogens will have a decreased plasma carnitine level (56), which may explain the sex differences of carnitine in human adults. Infant plasma carnitine levels are similar between males and females (57). Diet may also affect the level of plasma carnitine. Chronic starvation (58) is also associated with a decrease in plasma carnitine levels in humans. In guinea pigs, a diet deficient in ascorbic acid reduced the muscle carnitine concentration (58) and in newborn rats, a diet deficient in iron decreased the plasma carnitine concentration (43).

2. Carnitine Deficiency

Carnitine deficiency has been proposed as an etiology of endocardial fibroelastosis in infants and children (59), and in some

cases of dilated cardiomyopathies (60), lipid storage diseases (61,62), and non-ketotic hypoglycemia (63,64) because of defects in β-oxidation. Depressed levels of plasma carnitine are seen in Fanconi Syndrome due to insufficient reabsorption of carnitine (65), and in chronic renal dialysis patients due to loss of carnitine into the dialysate (66,67).

3. Carnitine in Pregnancy

Pregnant women have lower plasma carnitine concentrations [17.40 ± 1.23 nmol/ml (68), and 17.95 ± 1.00 nmol/ml (69)] compared with non-pregnant women [46.5 ± 12.4 nmol/ml (55)]. Hypertensive pregnant females have slightly higher levels of total carnitine. Pregnant diabetics have similar total carnitine levels than non-diabetics, however they have significantly lower concentrations of acylcarnitine. In all pregnant women, the concentration of plasma carnitine tends to decrease with advancing gestation, with lowest concentrations reported in mothers giving birth to term infants (69).

4. Infant Carnitine

Umbilical cord and infant plasma concentrations of carnitine are directly related to maternal levels of carnitine (68). The plasma carnitine level is higher in premature than in term neonates, 29.0 ± 1.8 (mean ± SEM) versus 22.4 ± 0.8 nmol/ml ($p<0.001$) (57). The mean plasma carnitine value in the unfed neonate is unchanged at 4 hours of age (68). However, plasma carnitine concentrations rapidly decrease in premature newborns during the first 3 days after birth if no exogenous carnitine is given (70).

J. Tissue Carnitine

Skeletal muscle tissue carnitine concentrations, unlike plasma levels, are lower in preterm compared to term infants (71,72) and adults (71). There is a positive relationship between muscle carnitine and gestational age ($r=0.832$, $p<0.001$) and birthweight ($r=0.746$, $p<0.001$). However, no correlation was found between gestational age or weight with liver and heart carnitine concentrations (71). Upon initiation of feedings, tissue carnitine levels increase in infants given a carnitine-containing formula or intravenous carnitine supplement. Infants maintained on parenteral alimentation for more than 10 days had lower heart, liver and kidney carnitine concentrations than controls (infants who died before 24 hours after birth) (72). Shenai and Borum (71) concluded that the inverse proportion of plasma and tissue carnitine levels at birth probably reflect enhanced placental acquisition or decreased tissue uptake of carnitine in the premature infant. They concluded from this observation that the premature infant is at greater risk for developing carnitine deficiency in the postnatal period.

K. Relationship of Plasma Carnitine to Diet

As mentioned, the infant plasma carnitine level depends on an exogenous source of carnitine. In infants, all fractions of carnitine decrease on a virtually carnitine-free diet and increase when carnitine is present in the diet (73). In weanling rats, dietary manipulation will change the ratio of non-esterified carnitine to acylcarnitine

(NEC:ASAC ratio), without changing the concentration of total plasma carnitine. Starvation or diets high in medium chain triglycerides or total fat will decrease the NEC:ASAC ratio (74). In older premature infants, administration of parenteral lipid emulsion caused an increase in acylcarnitine while total carnitine was maintained (75). However Yeh et al. (76) found that in younger premature infants there was a small insignificant increase in acylcarnitine during administration of fat emulsion. The premature infants exhibited lower ASAC levels than the term infants during a 5-9 day course of parenteral nutrition. The levels of β-hydroxybutyrate were found to be lower, while NEFA were significantly higher in the premature infants maintained on a total parenteral nutrition regime (which included lipid emulsion) when compared to term infants.

L. Relationship of Plasma Carntitine and Lipid Concentrations

Schmidt-Sommerfeld et al. (75) reported a positive relationship between total carnitine and β-hydroxybutyrate and between acylcarnitine and β-hydroxybutyrate ($r=0.45$, $p<0.05$, and $r=0.79$, $p<0.001$, respectively). Also, there was a negative relationship between plasma levels of total carnitine and NEFA ($r=-0.47$, $p<0.025$)(57), and between non-esterified carnitine and NEFA levels ($r=-0.653$, $p<0.01$)(24). From these results, both Yeh and Schmidt-Sommerfeld attributed carnitine insufficiency to the inability of the premature infant to adequately metabolize the parenteral lipid as an energy source.

M. Carnitine Supplementation

Intravenous carnitine supplementation produces a rapid rise in plasma free carnitine (77). Two hours after a 100 mg/kg dose of L-carnitine in neonates, plasma free carnitine increased to 1108.1 ± 132.2 nmol/ml. Acylcarnitines were also increased, showing that the infused carnitine is acylated in the neonatal tissues. However in the same study, there was no difference in lipid tolerance when a 1 g/kg dose of lipid emulsion was administered simultaneously with the carnitine supplement.

The effectiveness of intravenous carnitine supplementation to promote adequate fat metabolism has not been determined. The few studies which have been done in infants utilized only short term doses of supplemental carnitine. Orazali et al. (78) administered intravenous D,L-carnitine at an initial dose of 100 mg/kg followed by a continuous dose of 100 mg/kg over 6 hours with a similtaneous low dose (1 g/kg) of intravenous lipid emulsion. The subjects were older premature infants, having a mean gestational age of 36 weeks. After carnitine administration, the plasma ketone, NEFA and glycerol concentrations were all elevated when compared to the controls who did not receive carnitine supplementation. Triglyceride levels were not different between the groups during or after the infusion period. However, considering the older gestational age of the infants, the small lipid dose administered, and the short term administration of the carnitine supplement, no firm conclusions can be applied to the very low birthweight infant.

Studies are not available relating long term intravenous (or oral) carnitine therapy and lipid tolerance in infants. Investigators have shown that adults with Type IV hyperlipoproteinemia and adults and children on chronic hemodialysis have lower serum triglyceride levels when placed on long term oral carnitine supplementation (79,80,81,82). Several of the reported cases of systemic carnitine deficiency have also responded to carnitine supplementation (61,62).

Since premature infants appear to be at risk for developing both fat intolerance and carnitine deficiency, a relationship may exist between plasma levels of fat and carnitine.

CHAPTER III

EXPERIMENTAL PROCEDURE

This study included 46 appropriate for gestational age (AGA) infants born in, or admitted to, the Department of Pediatrics at the University of Tennessee Memorial Research Center and Hospital. The study was approved by the University of Tennessee Center for Health Sciences Institutional Review Board for Human Studies. Informed consent was required from a parent (or guardian) before an infant was included into the study. The subjects were classified into one of four groups based on birthweight. The four groups were: Group I, <1000 g; Group II, 1001-1510 g; and, Group III, 1511-2500 g; and Group IV, term AGA infants which served as the reference group for birth values. In the study, the infant birthweight ranged from 570 to 4536 g and the gestational age ranged from 24 to 42 weeks (Table 1).

Infant diets were ordered at the discretion of the attending staff physicians. Diets included both carnitine-free (<1 nmol carnitine/ml) and carnitine-containing formula/infusates. Carnitine-free diets consisted of total parenteral nutrition (TPN) which contained dextrose (varying concentrations) and crystalline amino acids, Aminosyn (Abbott Labs, North Chicago, Il.),intravenous lipid emulsion (Liposyn, Abbott Labs), and a commercial soy protein-based formula, Portagen (Mead Johnson Labs). Carnitine-containing diets included Enfamil-20 (20 kcal/oz), Enfamil-Premature Formula (Mead Johnson Labs), and breastmilk. The infants were placed on any number of these diets during

hospitalization. There was no particular order in which any infant or group received a particular type of diet.

Table 1. Weight and Gestational Age of Infants at Birth

Group	n	Weight (g)	Gestational Age (weeks)
I	10	780 ± 43.72[1]	26.5 ± 0.53
II	10	1350 ± 44.64	31.1 ± 0.53
III	16	1903 ± 61.45	33.1 ± 0.38
IV	10	3391 ± 184.54	39.8 ± 0.49

[1] Mean ± SEM

Blood samples (approximately 1 ml) were obtained for carnitine and lipid analysis from the mother within 16 hours after delivery and from umbilical cord within 4 hours after delivery. An sample of blood for routine hospital testing was also obtained from each infant soon after birth. Subjects in Groups I-III had daily blood samples drawn on days 0,1,2,3,4,5 and 7 of life, and then only prior to and after nutritional intervention, or weekly until discharge. All blood was collected in heparinized test tubes and centrifuged immediately at 4°. Plasma was separated and frozen at -20° until analyzed.

Plasma triglyceride and carnitine (non-esterified carnitine, acid-soluble acylcarnitine, and acid-insoluble acylcarnitine) levels were determined for each sample. Non-esterified fatty acid levels were measured on a portion of the samples obtained from Group I infants. Samples obtained following whole blood transfusions were excluded from

statistical analysis.

A. Plasma Triglyceride Determination

Plasma triglyceride (TG) was determined on each sample using the procedure of Giegel (83). The TG assay is based on the principle that plasma lipids may be extracted with nonane. The extracted TG are transesterified to yield three ethyl esters (soaps of fatty acids) and glycerol. The resulting glycerol is then oxidized to formaldehyde by periodate. Condensation of formaldehyde with ammonium ion and 2,4-pentadione will produce 3,5-diacetyl-1,3-dihydrolutidine which is a colored compound and can be measured spectrophotometrically at 415 nm.

The method was modified to accomodate a 50 µl plasma sample size. When tested, the modified technique resulted in 101.8% recovery compared to the standard technique described by Giegel.

1. Reagents

1. Extraction reagent: n-nonane (Fisher Scientific, Fairlawn, NJ) was mixed with isopropanol to obtain a 2.0/3.5 (v/v%) mixture.

2. Triolein standard (100mg/dl): 5.4 mg triolein (Sigma Chemical Co., St. Louis, MO) was added to 5.4 ml of extraction reagent (#1), mixed, and stored at -20°.

3. Sulfuric acid (40 mM H_2SO_4): 0.221 ml of concentrated H_2SO_4 was added to 100 ml of glass distilled water and the volume was brought to 500 ml with glass distilled water.

4. Transesterifying reagent: sodium hydroxide (100 mM) in isopropanol: 0.40 g NaOH was dissolved in approximately 80 ml of isopropanol and was brought to volume with isopropanol in a 100 ml volumetric flask.

5. Acetic acid (2 M CH_3CO_2H): 22.88 ml of glacial acetic acid was added to approximately 150 ml of glass distilled water and the volume was brought to 200 ml with glass distilled water.

6. Oxidizing reagent: sodium periodate (18 mM) in acetic acid: 0.3850 g sodium periodate was dissolved in approximately 80 ml of 2 M CH_3CO_2H (#5), mixed, and the volume was brought to 100 ml with 2M CH_3CO_2H.

7. Color buffer: ammonium acetate (6M CH_3COONH_4): 462.48 g CH_3COONH_4 was dissolved in approximately 700 ml of glass distilled water. The pH was adjusted to 6.0 with glacial acetic acid, and the volume was brought to 1 liter with glass distilled water.

8. Acetylacetone (2,4-pentadione): reagent grade, (Fisher Scientific, Fairlawn, NJ).

9. Color reagent: acetylacetone in color buffer: 4 ml of acetylacetone (#8) was added to each 100 ml of color buffer (#7) and mixed vigorously and allowed to stand at least 15 minutes before use. This solution is stable for 8 hours at room temperature and was made fresh before each assay.

2. Procedure

Extraction of triglyceride was accomplished by adding triolein standard (0, 0.02, 0.05, 0.1, 0.2 and 0.3 ml) to 100 x 75 mm glass test

tubes containing extraction reagent (2.50, 2.48, 2.45, 2.40, 2.30 and 2.20 ml, respectively), 0.1 ml glass distilled water and 0.5 ml H_2SO_4. Sample unknowns were prepared by adding 0.05 ml plasma to 2.45 ml extraction reagent, 0.1 ml glass distilled water and 0.5 ml H_2SO_4. All standard and sample tubes were vortexed and centrifuged at 4° for 10 minutes at 1500 x g, using a Beckman centrifuge (Beckman Instruments, Palo Alto, CA). A 0.25 ml portion of the upper nonane phase was obtained from standard tubes and two 0.25 ml portions were obtained from unknown tubes. A volume of 25 ml of transesterifying reagent was added, tubes vortexed and allowed to stand at least 5 minutes at room temperature. At this time 0.25 ml of oxidizing reagent was added to each tube, tubes were vortexed, and then allowed to stand at room temperature for at least 2 minutes, at which time 3.0 ml of working color reagent were added to each tube. All tubes were then capped, vortexed, and placed in a 56-60° water bath for 10 minutes to allow development of color. Once removed, the tubes were cooled to room temperature and absorbance was determined in a spectrophotometer (Beckman Model 34, Beckman Instruments, Fullerton, CA) at 415 nm. The concentration of known standards was plotted against absorbance and a standard curve was constructed. The inverse slope of the standard curve was estimated from the standard plot.

Triglyceride concentration of the unknowns was determined as follows:

$$\text{Triglyceride (mg/ml)} = A(\text{unknown}) * IS * df$$

where: A = absorbance at 415 nm,
IS = inverse slope of the standard curve, and
df = dilution factor (= 1000).

B. Plasma Carnitine Determination

Plasma carnitine concentration was determined by the radioisotopic procedure of Cederblad and Lindstedt (84), and as modified by others (85). The assay is based on the principle that non-esterified (free) L-carnitine will react with [1-^{14}C]acetyl CoA in the presence of carnitine acetyltransferase with subsequent formation of [1-^{14}C]acetylcarnitine. Placement of the mixture on anion exchange resin will exchange chloride ions for excess CoA. Therefore the eluate contains only labelled acetylcarnitine, which is evaluated in a liquid scintillation counter.

The standard assay used regularly in our lab utilizing a 100 µl sample size was modified to accomodate a 50 µl sample volume. When tested, the modified technique resulted in 95.4% recovery compared to the standard assay.

1. Reagents

1a. Carnitine Standard Solution (0.5 mM): 0.988 g of L-carnitine HCl (Sigma Chemical Co., St. Louis, MO) was dissolved in cold glass distilled water and the total volume was brought to 100 ml with glass

distilled water in a volumetric flask. One ml portions were dispensed into plastic tubes and frozen a -70°. Each portion was diluted appropriately (0.25 mM) for the working standard solution.

1b. Palmityl Carnitine Standard (22.9 mM): 1.0 ml of glass distilled water was added to a vial containing 10 ml of L-palmityl carnitine (Sigma Chemical Co., St. Louis, MO). This was diluted appropriately (0.229 mM) for the working standard solution.

1c. Carnitine Standard Mixed Solution: 1.0 ml of 0.25 mM L-carnitine HCl was added to 1.0 ml of 0.229 mM L-palmitylcarnitine, mixed, and stored frozen at -20°.

2. Potassium Hydroxide (0.5 N KOH): 28.055 g KOH pellets were dissolved in glass distilled water and the total volume was brought to 1 liter with glass distilled water.

3. Perchloric Acid (0.6M): 51.26 ml of 70% Perchloric acid was added to 500 ml of glass distilled water and the total volume was brought to 1 liter with glass distilled water.

4. Sodium Tetrathionate (0.1 mM $Na_2S_4O_6$): 0.7656 g of $Na_2S_4O_6$ was dissolved in glass distilled water and made to volume in a 25 ml volumetric flask.

5. Phenol Red (0.1%): 0.1 g of Phenol Red was dissolved in 100 ml of absolute ethanol. (Phenol Red tubes were made by adding one drop of Phenol Red indicator to 12 x 75 mm Pyrex glass tubes and allowed to dry).

6. Radioactive Acetyl Coenzyme A (50 mCi/mM): 50 µCi of [1-^{14}C]acetyl coenzyme A (Amersham Corp., Chicago, IL) was dissolved in 300 ml of cold glass distilled water. Portions of approximately 9 ml

were dispensed into plastic vials and frozen at -60°.

7. Potassium Bicarbonate (1 M $KHCO_3$): 1.0 g of $KHCO_3$ was dissolved in glass distilled water and the total volume was brought up to 10 ml in a graduated conical test tube.

8. Acetic Anhydride [0.1 M $(CH_3CO)_2O$]: 0.5 ml of $(CH_3CO)_2O$ was added to 4.95 ml of cold glass distilled water, mixed, and used immediately for the acetyl CoA solution.

9. Acetyl Coenzyme A (0.1 mM): 10 mg of coenzyme A (Sigma Chemical Co., St. Louis, MO) was dissolved in 0.5 ml of cold glass distilled water. To this, 100 µl of 1 M $KHCO_3$ was added. Then, 200 µl of 0.1 M acetic anhydride was added and the volume was brought to 80 ml with cold glass distilled water. After mixing, the solution was dispensed into plastic tubes in approximately 8 ml portions and stored at -60°.

10. Carnitine Acetyltransferase (Pigeon Breast Muscle): acetyl CoA: carnitine-O-acetyltransferase (Sigma Chemical Co., St. Louis, MO) was diluted with glass distilled water to 50 units/ml, gently mixed, then refrigerated in approximately 1 ml portions until use.

11. MOPS Buffer (1 M [3-{4-morpholino}propanosulfonic acid]): 20.92 g of MOPS (Sigma Chemical Co., St. Louis, MO) was dissolved in approximately 80 ml of glass distilled water. The pH was adjusted to 7.4 with 4 N KOH, and the volume was brought up to 100 ml with glass distilled water.

12. PCA/MOPS I: 20.9 g of MOPS was added to 20 ml of 0.6 M perchloric acid and brought to 100 ml with glass distilled water.

13. PCA/MOPS II: 20.9 g of MOPS was added to 20 ml of 0.6 M perchloric and and brought to 100 ml with glass distilled water.

14. EGTA (0.1 M, pH 7.0): 1.902 g of EGTA [Ethyleneglycol-bis(B-amino-ethylether)N,N'-tetraacetic acid] (Sigma Chemical Co., St. Louis, MO) was dissolved in 30 ml of glass distilled water and neutralized with 4 N KOH to pH 7.0. The volume was then made to 50 ml with glass distilled water in a volumetric flask.

15. Radioactive Acetyl CoA mix (0.1 mM [1-^{14}C]acetyl CoA solution: 2 volumes of [1-^{14}C]acetyl CoA (#7) were mixed with 1 volume of 0.1 mM acetyl CoA (#10) and kept on ice.

16. Reagent mixture for each assay:

MOPS (0.1 M) buffer pH 7.4 (#12)	120 µl
EGTA (0.1 M) pH 7.0 (#15)	20 µl
$Na_2S_4O_6$ (0.1 M) (#5)	20 µl
Radioactive acetyl CoA mix (0.1 M) (#16)	200 µl
Glass distilled water	40 µl

17. Bovine serum albumin (8% Fraction V, fatty acid poor from ICN Nutritional Biochemicals, Cleveland, OH): 4 g of BSA was dusted into about 20 ml of glass distilled water and stirred. The volume was made to 50 ml with glass distilled water in a volumetric flask.

18a. Scintillation fluid A: 33 g of PPO (2,5-diphenyloxazole) and 1 g of POPOP (1,4-Bis[2-{5-Phenyloxazolyl}benzene]) were dissolved in 4 liters of toluene ($C_6H_5CH_3$).

18b Scintillation fluid B: 2 parts of scintillation fluid A (#18a) was mixed with 1 part Triton X-100 (Fisher Scientific Co., Fair Lawn, NJ).

2. Procedure

Carnitine standards and samples were prepared in 12 x 75 mm Pyrex glass test tubes as shown in Table 2. Standard and sample tubes were vortexed and then centrifuged at 4° for 10 minutes at 1500 x g using a Beckman centrifuge (Beckman Instruments, Palo Alto, CA). The supernatant fluid was used to quantitatively determine the concentrations of non-esterified carnitine (NEC), and acid-soluble acylcarnitine (ASAC). The ASAC fraction was calculated by subtracting NEC from the total ASAC. The pellet was used to quantitate the concentration of acid-insoluble acylcarnitine (AIAC). The NEC, ASAC, and AIAC are equivalent to the free carnitine, short-chain acylcarnitines, and the long-chain acylcarnitines respectively, described in the literature.

Table 2. Standard Mixtures of Carnitine

CNE-std[1] (µl)	L-CNE[2] (nm)	P-CNE[3] (nm)	GDW[4] (µl)	8% BSA[5] (µl)	0.6 M PCA[6] (µl)	Plasma (µl)	Total (µl)
0	0	0	100	100	200	0	400
10	1.25	1.15	90	100	200	0	400
20	2.50	2.30	80	100	200	0	400
30	3.75	3.45	70	100	200	0	400
40	5.00	4.60	60	100	200	0	400
60	7.50	6.90	40	100	200	0	400
0	0	0	125	25	200	50	400

1 carnitine standard mix
2 L-carnitine (non-esterified)
3 L-palmitoylcarnitine
4 glass distilled water
5 bovine serum albumin
6 perchloric acid

a. Non-esterified Carnitine (NEC) Determination

A 150 µl portion of the PCA supernatant fluid was transferred to 12 x 75 mm phenol red pyrex test tubes and neutralized with 35 µl 1 M $KHCO_3$. The tubes were placed on ice and allowed to stand at least 30 minutes until ready to assay for carnitine.

b. Acid-soluble Acylcarnitine (ASAC) Determination

A 100 µl portion of the PCA supernatant was transferred to 12 x 75 mm phenol red Pyrex test tubes. Alkaline hydrolysis was achieved by adding 75 µl of 0.5 N KOH and then incubating at 37° for 30 minutes. Tubes were then neutralized with 30 µl PCA-MOPS II, then placed on ice for 30 minutes until ready to assay for carnitine.

c. Acid-insoluble Acylcarnitine (AIAC) Determination

The pellets were drained of any remaining PCA supernatant and washed twice by resuspending in 0.6 M PCA. An intact pellet was regained after each wash by centrifuging at 1500 x g for 10 minutes. One drop of phenol red indicator was placed in each tube. Alkaline hydrolysis of the the PCA supernatant-free pellets was accomplished by adding 200 µl 0.5 N KOH and then incubating in water bath at 65° for 60 minutes. Tubes were then neutralized with 100 µl PCA-MOPS I and the tubes were then placed on ice at least 30 minutes until ready to assay for carnitine.

d. Assay for Carnitine

A 100 µl portion was transferred from each of the appropriate fractions for non-esterified carnitine, acid-soluble acylcarnitine and

acid-insoluble acylcarnitine into 1.5 ml capacity microtubes. Four hundred µl of reagent mixture and 20 µl (1 Unit) of carnitine acetyltransferase were added to initiate the enzymatic reaction for [1-^{14}C]acetylcarnitine formation. The tubes were capped, gently mixed by agitation, then placed in a 37° water bath for a 30 minute incubation period. After this, a 200 µl aliquot of the incubation mixture was placed on a 4.5 cm anion exchange minicolumn (columns were made by packing Dowex 1 x 8, 200-400 mesh, Cl^- into a 0.5 x 5.75 inch glass pasteur pipette). After the sample was adsorbed on the anion exchange resin, the columns were eluted with 2 portions of 500 µl glass distilled water into a 20 ml-capacity scintillation vial (New England Nuclear, Boston, MA). The eluate was mixed with 10 ml of scintillation fluid B, capped, gently mixed by swirling, and then placed into a liquid scintillation counter (Beckman models LS 100C and/or LS 3801, Beckman Instruments, Palo Alto, CA) to evaluate the radioactivity in each vial. The concentration of the carnitine standards was plotted against the radioactive counts for each standard. A standard curve was plotted and the inverse slope of the standard curve was estimated from this plot.

Carnitine concentration of each fraction was determined as follows:

$$\text{Carnitine concentration (nmol/ml)} = IS * (CPM_{sample} - CPM_{blank}) * df$$

where: IS = inverse slope of the standard curve,
CPM = counts per minute, and
df = dilution factor (= 20).

Total Carnitine (TC): the total carnitine concentration was the mathematical sum of the three fractions of carnitine, i.e.,

$$TC = NEC + (\text{total ASAC} - NEC) + AIAC.$$

C. Infant Formula Carnitine Determination

The carnitine content of Portagen, Enfamil-Premature Formula, and Enfamil-20 were determined according to the procedure for plasma carnitine determination. The only modifications were: (1) an appropriately diluted 100 µl sample, instead of the 50 µl plasma sample, and (2) the determinations were done in triplicate. The average carnitine concentration used for calculations of carnitine intake.

D. Plasma Non-Esterified Fatty Acid Determination

The determination of plasma non-esterified fatty acids was based on the method of Novak (86). The plasma NEFA may be extracted with a mixture of isopropyl alcohol and heptane. The extracted lipids will react with cobalt to form cobalt soaps. The cobalt soaps will give a color reaction when mixed with α-nitroso-β-naphthol which can be measured spectrophotometrically at 500 nm.

1. Reagents

1. Saturated K_2SO_4 solution: K_2SO_4 was added to boiling water until saturated. The solution was stored in contact with the extra crystals overnight, and then filtered.

2. Cobalt nitrate-acetic acid-potassium sulfate solution: 6 g of $Co(NO_3)_2 \cdot 6H_2O$ and 0.8 ml of glacial acetic acid were mixed with the

saturated K_2SO_4 solution (#1), and the volume was brought to 100 ml in a volumetric flask. The contents were stored at 37°.

3. Saturated Na_2SO_4 solution: Na_2SO_4 was added to boiling water until saturated. The solution was stored in contact with the extra crystals overnight, and then filtered.

4. Cobalt reagent: 1.35 volumes of triethanolamine, 10 volumes of the cobalt nitrate-acetic acid-potassium sulfate solution, and 7 volumes of the saturated Na_2SO_4 solution were mixed together in a glass beaker. This reagent was made fresh every series of analyses.

5. Indicator solution (stock solution): 400 mg of α-nitroso-β-naphthol was dissolved in 96% ethanol. A working solution was made fresh for each series of analyses by diluting the stock solution by a factor of 12.5 before use.

6. Dole's extraction mixture: 40 volumes of redistilled isopropyl alcohol, 10 volumes of redistilled heptane, and 1 volume of 1 N H_2SO_4 were mixed together and the contents were stored at room temperature.

7. Palmitic acid standard (0.4 meq/dl): 10.24 mg of palmitic acid was dissolved in 100 ml of Dole's extraction reagent and was stored at -20°.

8. Chloroform-Heptane mixture: 5 volumes of redistilled chloroform was mixed with 1 volume of redistilled heptane and stored at 20°C until use.

2. Procedure

The blank and standard test tubes were prepared by adding 250µl of Dole's extraction reagent or 250 µl of palmitic standard to their

respective tubes. A volume of 50 µl of glass distilled water was added to each of the blank and standard tubes. Samples of unknown NEFA concentrations were prepared by adding 50 µl of plasma to 250 µl of Dole's extraction reagent. All tubes were vortexed and then placed in melting ice water for 10 minutes. A volume of 400 µl of heptane and 500 µl of glass distilled water was added to each tube. All tubes were then vortexed to allow separation of phases. After this, 300 µl of the upper heptane phase was transferred to a 1.5 ml-capacity plastic microtube. This was followed by the addition of 500 µl of the chloroformnnn-heptane mixture and 500 µl of cobalt reagent. The tubes were placed in an automatic shaker and vigorously mixed for 3 minutes. The microtubes were then centrifuged at 1800 x g for 15 minutes. After centrifugation, 600 µl of the chloroform-heptane phase was added to 12 mm x 75 mm glass test tubes which contained 750 µl of the working indicator solution. The tubes were allowed to stand at room temperature for 30 minutes for color development. Next, the absorbance of each tube was read against the blank at 500 nm. A standard curve was plotted and the inverse slope was estimated from the standard curve. The quantification of NEFA of the plasma unknowns was determined as follows:

$$\text{NEFA (meq/l)} = A_{(\text{unknown})} * IS * df$$

where: A = absorbance at 500 nm,
IS = inverse slope of the standard curve, and
df = dilution factor (= 20).

E. Statistical Methods

The Students "t" test (87) was utilized to evaluate maternal, umbilical cord, and infant carnitine and triglyceride levels. Simple Regression, utilizing the method of regression of least squares was used to determine all correlation coefficients (r), and the significance was determined by an Analysis of Variance. A statistical software package (Statgraphics, Statistical Graphics Corporation) was used for all regression statistics. The Students "t" test for paired comparisons (88) was used to evaluate the effects of nutritional intervention on carnitine and triglyceride levels. For all data analyzed, the minimal level of statistical significant acceptance was $p<0.05$.

CHAPTER IV

RESULTS

A. Plasma Carnitine at Birth

1. Maternal, Umbilical Cord and Infant Plasma Carnitine Levels

The maternal, umbilical cord, and infant mean plasma carnitine and triglyceride concentrations obtained within 8 hours post-partum are presented by group in Figure 1 and in Tables 3 and 4. The values were compared between and within each group. Both paired and unpaired maternal, cord, and infant data are listed in the tables. Since paired data were limited (Table A-2), the unpaired data were incorporated into the text of the results and the discussion will be focused on the analysis of the unpaired data.

Maternal NEC and TC in Groups I and II were significantly higher when compared with Groups III and IV. There was no difference in maternal CNE between either Group I and II or Group III and Group IV (reference group). The maternal plasma levels of ASAC were not significantly different between the control and experimental groups. The maternal plasma levels of long-chain AIAC were similar in all groups except Group I, which had significantly higher AIAC levels compared to the other groups.

The umbilical cord levels of NEC, ASAC, and CNE were similar in all groups. However Groups I, II, and III had significantly higher mean levels of AIAC than the control group.

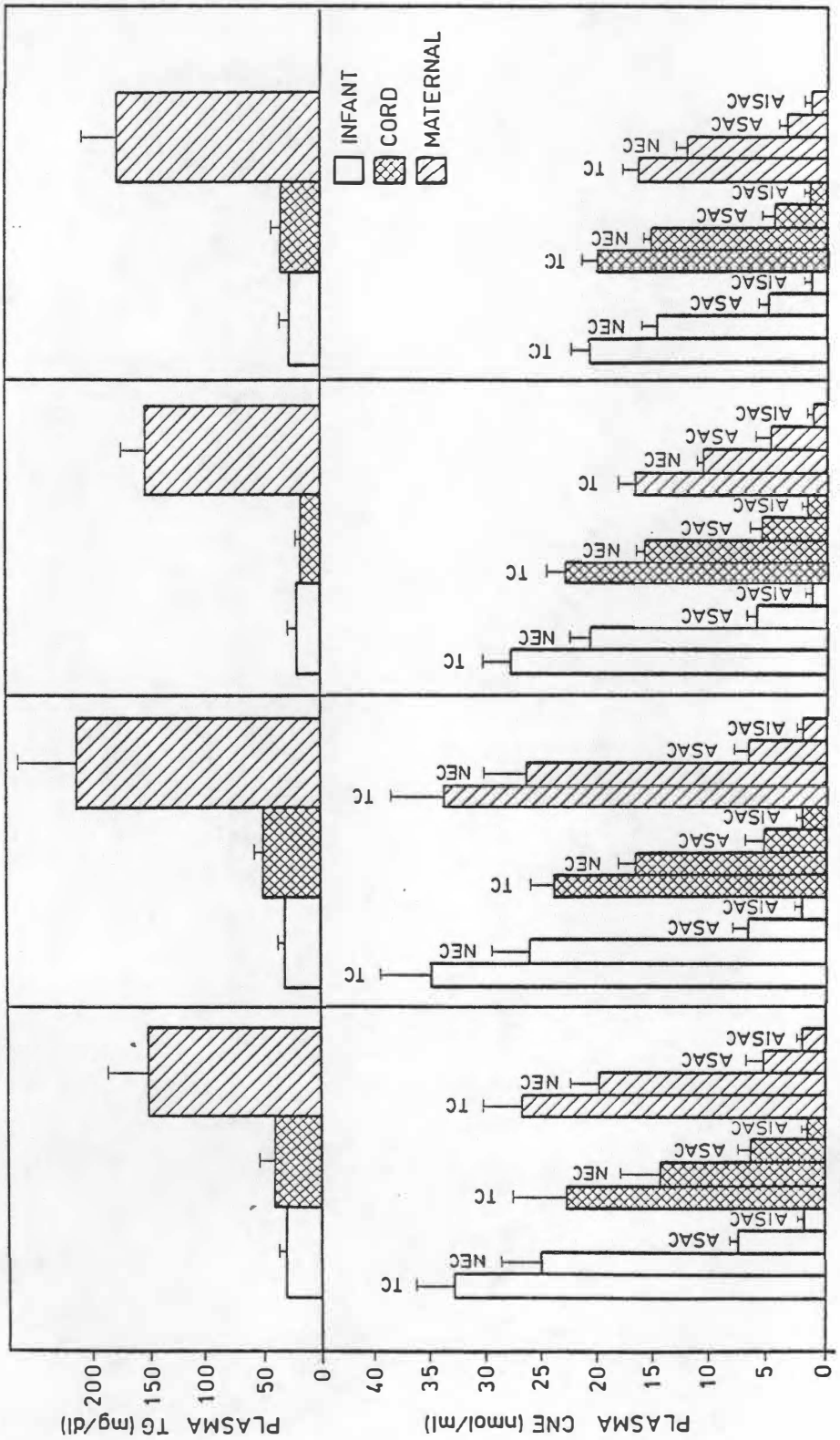

Figure 1. Maternal, Umbilical Cord, and Infant Plasma Carnitine and Triglyceride Levels at Birth

Table 3. Plasma Carnitine Levels[1,2] (nmol/ml) at Birth[3] for Maternal, Umbilical Cord, and Infant Plasma (Unpaired).

Group	Maternal		Cord		Infant	
Total Carnitine						
I	22.5 ± 3.3	a,f	21.9 ± 4.5	a,f	32.7 ± 3.4	ab,f
II	29.9 ± 4.5	a,fg	24.3 ± 1.9	a,g	36.1 ± 4.4	a,f
III	16.4 ± 1.6	b,g	22.7 ± 1.6	a,f	26.3 ± 2.2	bc,f
IV	16.4 ± 1.0	b,g	20.3 ± 1.1	a,f	21.0 ± 1.4	c,f
Non-Esterified Carnitine						
I	19.4 ± 2.4	a,fg	14.8 ± 3.5	a,g	25.3 ± 3.1	ab,f
II	22.7 ± 3.7	a,fg	16.5 ± 1.1	a,g	29.5 ± 3.0	a,f
III	10.7 ± 0.6	b,h	15.8 ± 3.2	a,g	20.2 ± 1.7	b,f
IV	12.2 ± 0.8	b,g	15.0 ± 0.7	a,f	15.2 ± 1.1	c,fg
Acid-Soluble Acylcarnitine						
I	5.5 ± 1.4	a,f	6.5 ± 1.1	a,f	6.4 ± 0.5	a,f
II	6.2 ± 1.1	a,f	6.3 ± 1.9	a,f	6.4 ± 1.2	a,f
III	4.7 ± 1.1	a,f	5.6 ± 0.8	a,f	5.3 ± 2.6	a,f
IV	3.6 ± 0.6	a,f	4.4 ± 1.0	a,f	5.2 ± 0.7	a,f
Acid-Insoluble Acylcarnitine						
I	1.6 ± 0.1	a,f	1.2 ± 0.1	a,g	1.0 ± 0.1	a,g
II	1.1 ± 0.1	b,f	1.5 ± 0.2	a,f	1.2 ± 0.2	a,f
III	1.0 ± 0.3	a,f	1.2 ± 0.1	a,f	0.9 ± 0.1	a,f
IV	0.8 ± 0.1	b,f	0.8 ± 0.1	b,f	0.7 ± 0.1	a,f

1 Values are the group means ± SEM

2 Means in a row or column without a common superscript letter are different ($p<0.05$)

3 Plasma samples were obtained within 8 hours post-partum

Table 4. Plasma Triglyceride Levels (mg/dl)[1,2] at Birth[3] for Maternal, Umbilical Cord, and Infant Plasma (Unpaired).

Group	Maternal	Cord	Infant
I	151 ± 33 [a,g]	39 ± 11 [a,f]	31 ± 5 [ab,f]
II	216 ± 51 [a,g]	36 ± 6 [a,f]	29 ± 3 [a,f]
III	168 ± 23 [a,g]	19 ± 2 [b,f]	19 ± 3 [b,f]
IV	175 ± 27 [a,g]	30 ± 5 [a,f]	26 ± 15 [ab,f]

[1] Values are the group means ± SEM

[2] Means in a row or column without a common superscript letter are different ($p<0.05$)

[3] Plasma samples were obtained within 8 hours post-partum

The infant plasma levels of NEC were higher in the premature infants compared with the term (Group IV) infants, and Groups I and II had significantly higher NEC levels than Groups III (the oldest premature infants). The plasma TC levels were highest in Group II, although Group I was not significantly different than Group II. Like NEC, the premature infants had higher TC levels than the controls, while only Groups I and II were significantly higher than the controls. There were no significant differences among groups for either ASAC or AIAC (Table 3).

Intra-group comparisons of maternal, umbilical cord, and infant carnitine are also presented in Table 3. In all groups, the infant NEC and TC levels were higher than the maternal and umbilical cord levels. Within a group, the differences of NEC were variable. There was not difference between the NEC levels of the maternal and umbilical cord or the umbilical cord and the infant. In Group III and IV, the NEC levels were significantly higher in umbilical cord and infant, compared to the maternal plasma. In Group IV, maternal and umbilical cord NEC levels were different ($p<0.05$) while infant levels of NEC were not different between the maternal and umbilical cord levels.

The maternal, umbilical cord, and infant ratio (M:C:I ratio) of TC within each of the three groups is presented in Table A-4. The ratio of maternal, cord, and infant carnitine values were not identical between any group. The ratio of maternal TC was higher than umbilical cord TC in Groups I and II, but in Groups III and IV the ratio of umbilical cord to maternal CNE was higher.

2. Relationship Between Gestational Age and Carnitine Levels at Birth

The relationship of infant plasma carnitine levels at birth compared to gestational age is presented in Figures 2 and 3. There was a decrease in plasma TC ($r=-0.4418$, $p<0.01$) with advancing gestational age. However, there was an increase in TC at birth ($r=0.7278$, $p<0.005$), in infants of gestational age 24.5-31.5 weeks. After this time the TC levels decreased to an apparent plateau at approximately 20 nmol/ml by 34 weeks. The changes in NEC paralleled those of TC. There was not a significant correlation of ASAC or AIAC with advancing gestational age.

B. Changes in Carnitine and Triglyceride With Postnatal Age

The mean carnitine and triglyceride levels with advancing gestational age are presented by group in Figures 4-6. During the first week post-partum, all three groups exhibited a decrease in NEC and TC levels. The greatest decrease occurred in Group II, followed by Group III, and the smallest decrease occurred in Group I. With time, all groups exhibited an increase in total carnitine levels. Group III achieved birth carnitine levels by day 7, Group II on day 28, and Group I on day 91. The increase in plasma carnitine coincided with the initiation of a carnitine-containing diet.

No infant received dietary carnitine before plasma samples were obtained for days 0 and 1. During the first 24 hours post-partum, the percent mean change in TC levels were as follows: Group I +5%, Group II -24%, and Group III -9%. By day 5, the percent changes of TC were -5%, -41%, and -7% for Groups I, II, and III, respectively. However, by day 7, the percent mean changes were -21%, -34%, and +15%, respectively.

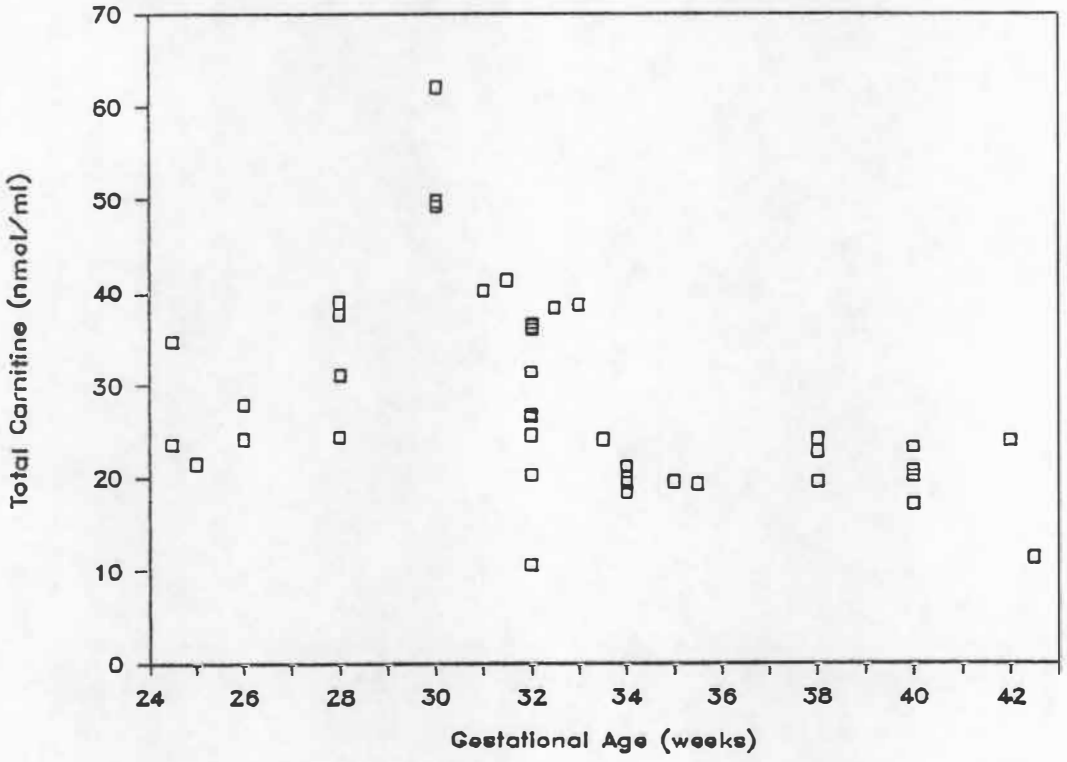

Figure 2. Relationship of Plasma Total Carnitine with Gestational Age of All Infants at Birth.

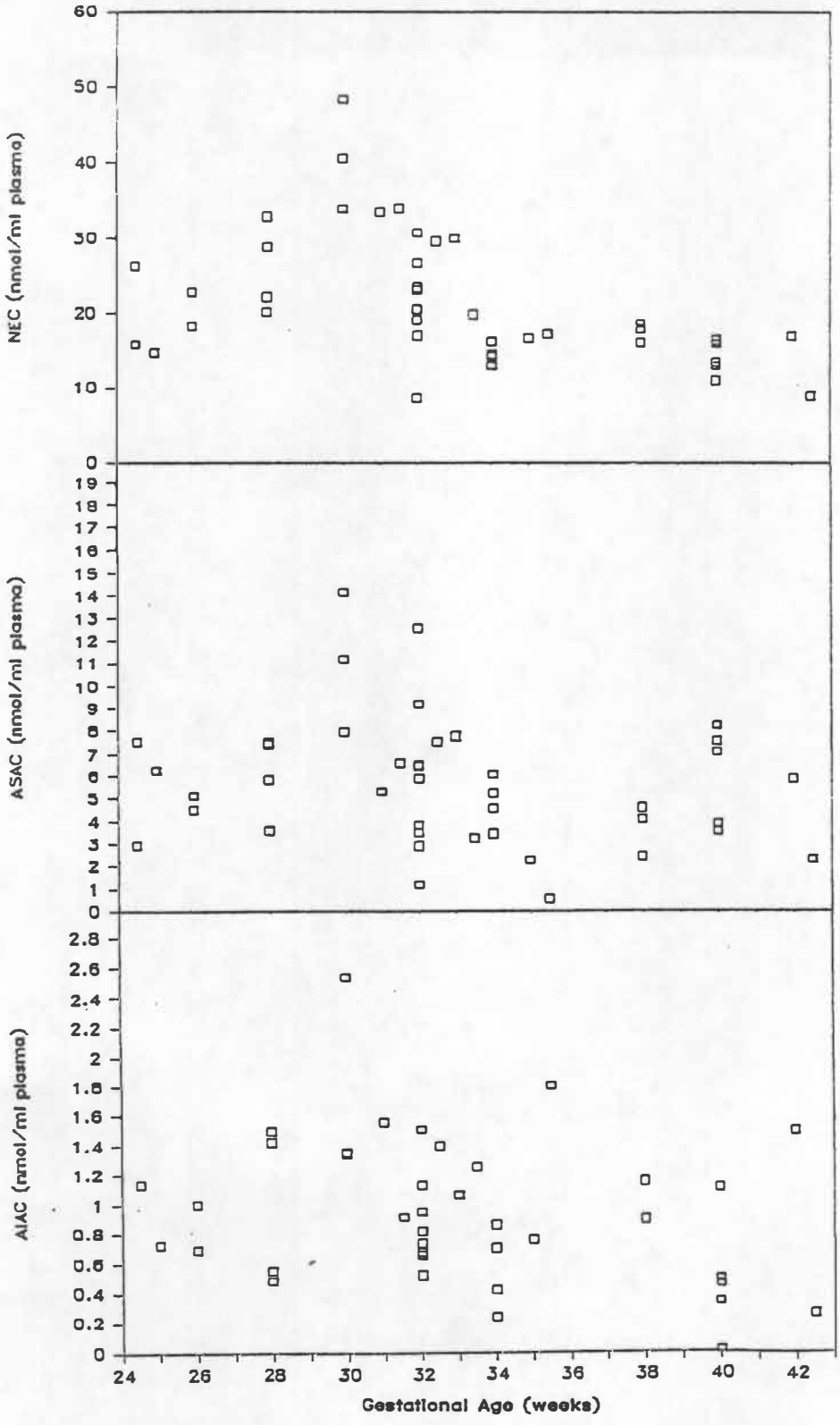

Figure 3. Relationship of NEC, ASAC, and AIAC with Gestational Age of All Infants at Birth.

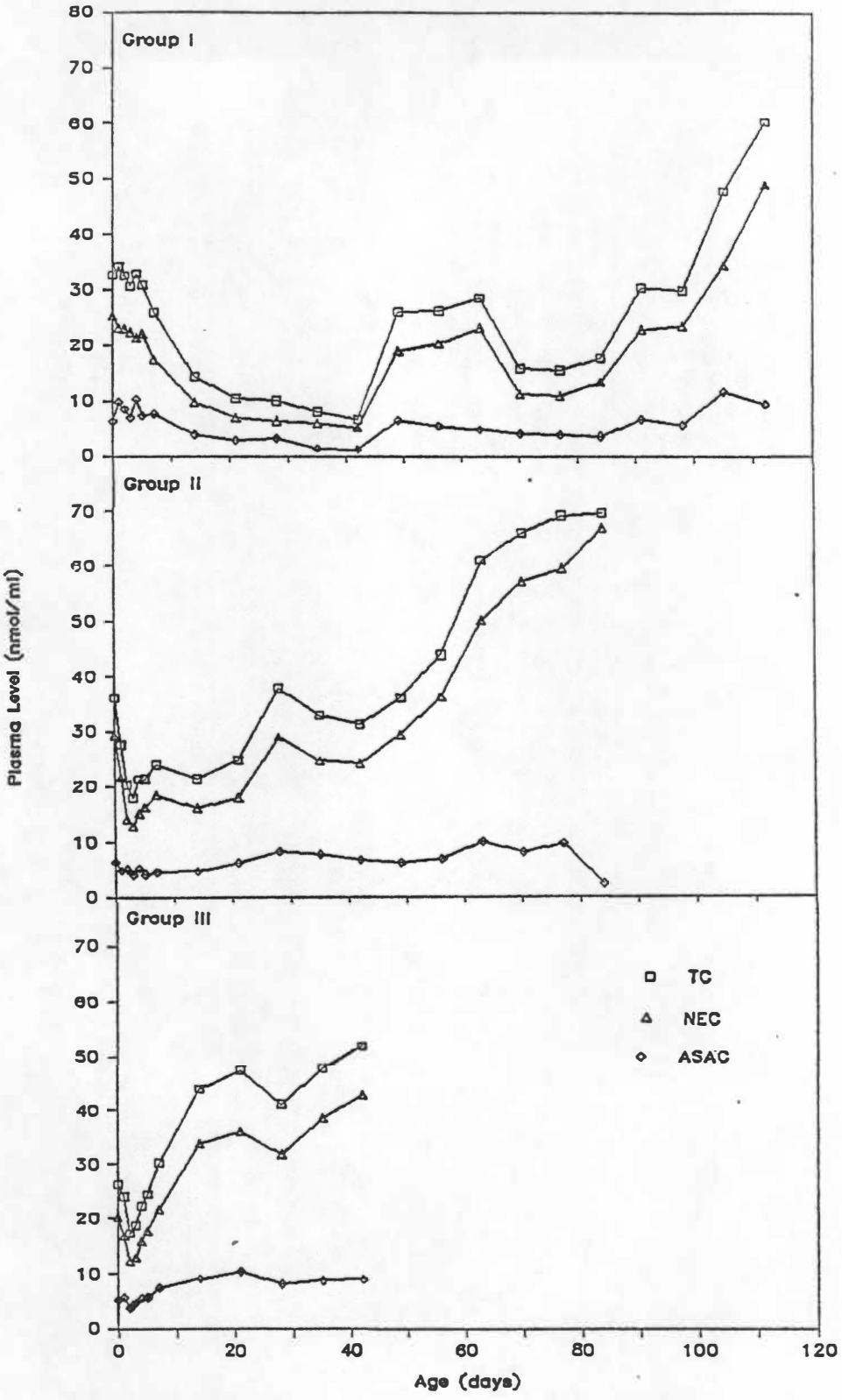

Figure 4. Postnatal Changes in TC, NEC, and ASAC of Infants in Groups I, II, and III.

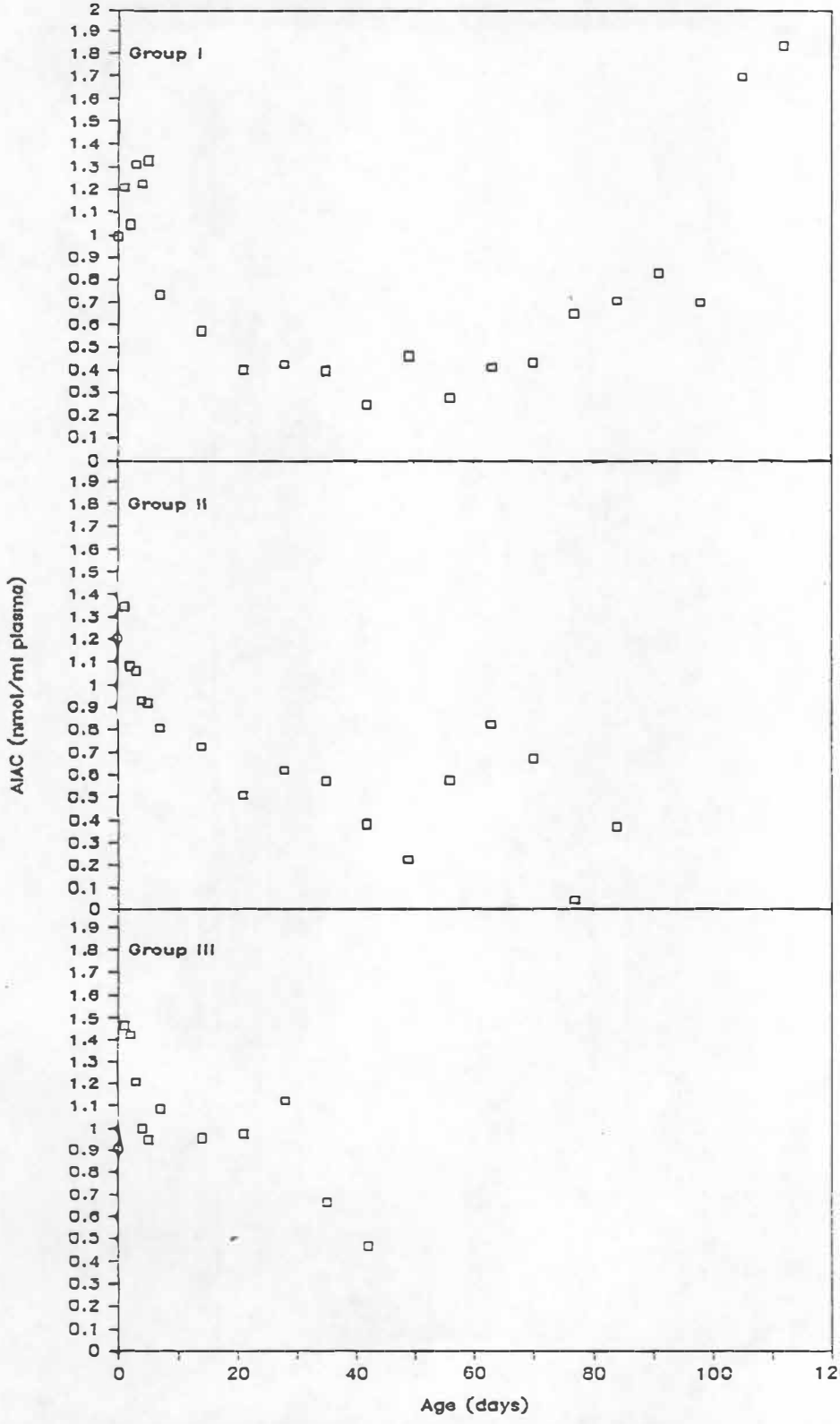

Figure 5. Postnatal Changes in AIAC of Infants in Groups I, II, and III.

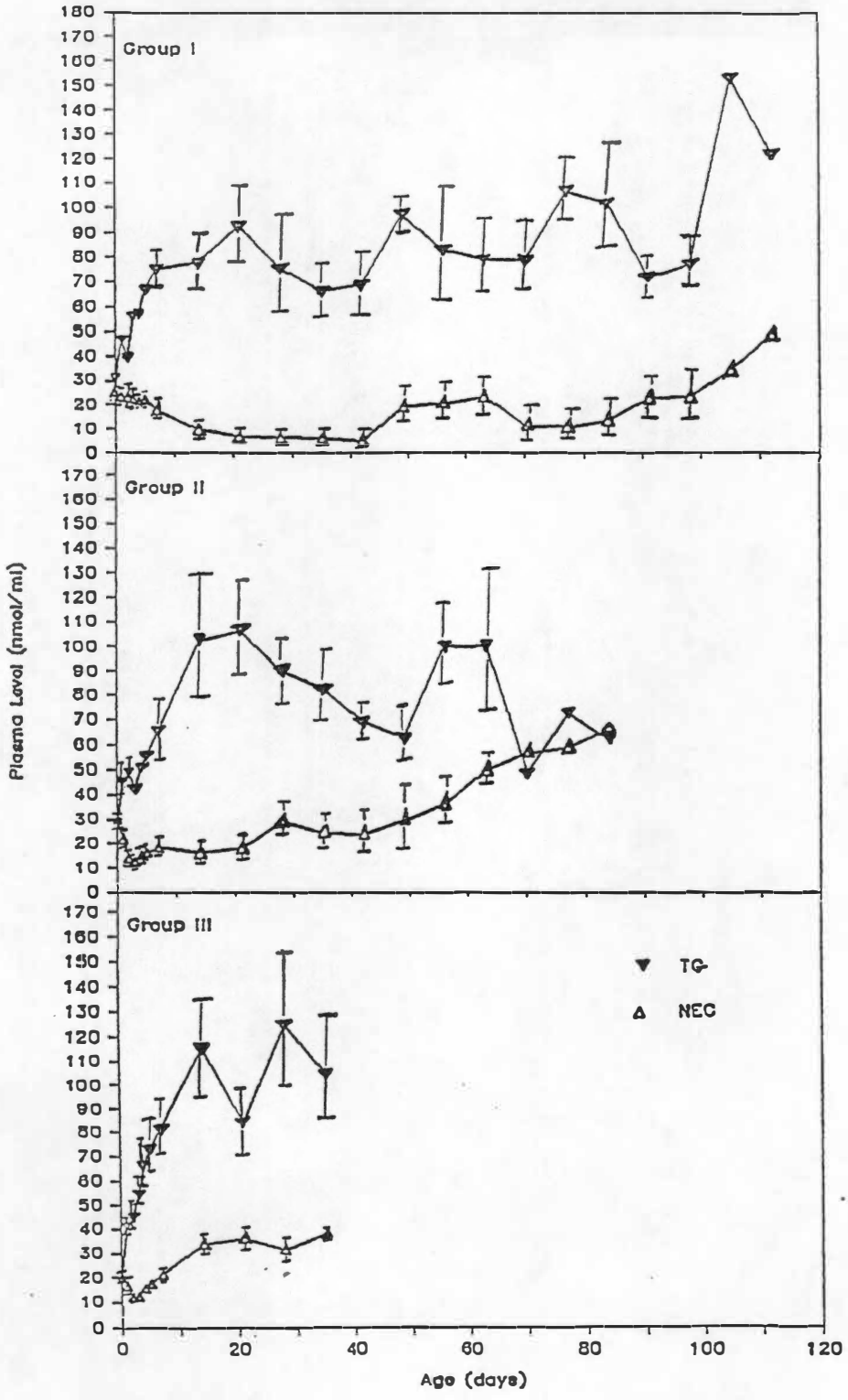

Figure 6. Postnatal Comparison of the Changes in NEC and TG of Infants in Groups I, II, and III.

After the first post-partum week, Group I infants continued to show a decrease in TC until day 42 at which time the TC level was 5.2 nmol/ml. These infants did not obtain birth TC levels until day 91 (30.4 nmol/ml). After this the TC level continued to rise and was 60.4 nmol/ml on day 112. Infants in Group II also showed a decrease in TC during the first week and then a rise until day 28 (28.6 nmol/ml). The TC level plateaued on day 28, then increased after day 56 to a final value of 69.4 nmol/ml on day 84. Infants in Group III continued to show a steady increase of TC from day 7 until day 21. After this, the level of CNE appeared to plateau at about 38-43 nmol/ml until all infants had been discharged on day 42.

In all groups, NEC paralleled the changes of TC (Figure 4). Since NEC and ASAC are influenced by the metabolic state, by the ability of the infant to utilize fat, and dietary factors, these levels are used together and stated as the NEC to ASAC ratio (the NEC:ASAC ratio was calculated from the data points shown in Figure 4). The daily NEC:ASAC ratio was similar in each group at birth (3.97, 4.58, and 3.83 in Groups I, II, and III, respectively) and also throughout the study. The NEC:ASAC ratio decreased in all groups during the first few days of life. The ratio remained below the birth value until days 56, 49, and 14 in Groups I, II, and III, respectively.

The group means of AIAC followed similar patterns throughout the study. All groups had similar AIAC levels at birth. The AIAC peaked within the first week of life in each of the three groups. AIAC in Groups II and III each peaked on day 1, while Group I AIAC began to increase on day 1, (AIAC did not peak until day 3). Group I retained

the peak levels longer, 3 days, while the other two groups had a peak which lasted only one day.

The changes in TG levels with advancing age showed a similar trend in all groups. The TG levels showed a continuous increase during the first 14 days. After this, TG levels remained relatively constant. The mean TG in Group I appeared to plateau at approximately 85 mg/dl, in Group II at 80 mg/dl (with more fluctuation than the other groups), and in Group III, at approximately 105-110 mg/dl.

C. Carnitine Content of Infant Formula

The results of the carnitine determination on the commercial infant formulas used in the study are presented in Table 5. There was no detectable carnitine in Portagen (soy protein-based formula). There were substantial amounts of carnitine in Enfamil-PF and in Enfamil-20 (both are milk-based formulas). The average total carnitine was 327 nmol/ml in Enfamil-PF and 184 nmol/ml in Enfamil-20.

D. Relationship of Plasma Carnitine and Triglyceride with Various Nutritional Interventions

1. Carnitine and Triglyceride Levels After Total Parenteral Nutrition

The changes in carnitine and triglyceride after total parenteral nutrition (TPN) are presented in Table 6 and Figure A-1. In all three groups, the concentrations of NEC, ASAC, AIAC, and TC decreased after infants had received TPN for 24 hours. Only Groups II and III had a significant decrease in NEC and TC. However, the mean decreases in NEC and TC were greater in Group I than in Group II or Group III. A

Table 5. Carnitine Content of Infant Formulas[1,2]

Carnitine Fraction	Portagen	Enfamil-PF	Enfamil-20
NEC	ND[3]	270 ± 1	155 ± 3
ASAC	ND	56 ± 3	34 ± 2
AIAC	ND	1 ± 0	0 ± 0
TC	ND	327 ± 3	184 ± 3

[1] Values are reported in nmol/ml of formula

[2] Values are the mean ± SEM

[3] Non-detectable

Table 6. Plasma Carnitine and Triglyceride Levels[1] Before, 24 Hours After Initiation, and Upon Discontinuation of Total Parenteral Nutrition.

Parameter	Group	n	Pre-TPN	24° Post-TPN	Post-TPN[2]
NEC[3]	I	8	24.9 ± 4.2	16.5 ± 2.9 [a]	4.9 ± 0.5 [a]
	II	10	18.6 ± 2.7	15.5 ± 1.9 [a]	9.8 ± 1.7 [a]
	III	9	15.7 ± 1.5	11.9 ± 1.4 [a]	13.8 ± 1.1
ASAC[4]	I	8	10.4 ± 2.3	6.6 ± 1.4	2.7 ± 0.5 [a]
	II	10	5.6 ± 1.0	5.9 ± 0.8	4.0 ± 0.8
	III	9	5.5 ± 0.5	3.2 ± 1.0 [a]	3.6 ± 0.5
AIAC[5]	I	8	1.3 ± 0.4	0.9 ± 0.1	0.5 ± 0.2 [a]
	II	10	1.1 ± 0.3	0.7 ± 0.1	0.7 ± 0.2
	III	9	1.6 ± 0.2	1.1 ± 0.2 [a]	0.8 ± 0.1
TC[6]	I	8	36.3 ± 6.5	24.1 ± 4.3	8.2 ± 0.7 [a]
	II	10	25.4 ± 2.2	21.6 ± 2.8 [a]	14.5 ± 2.4 [a]
	III	9	22.8 ± 2.4	16.3 ± 1.9 [a]	18.2 ± 1.4 [a]
TG[7]	I	8	55.6 ± 9.5	41.5 ± 3.7	84.0 ± 17.4
	II	10	51.2 ± 4.5	43.2 ± 4.9 [a]	58.0 ± 12.6
	III	9	49.1 ± 5.6	59.7 ± 7.8	55.0 ± 18.6
Fat Intake[8]	I	8	0.00 ±	0.00 ±	4.11 ± 0.24
	II	10	0.30 ± 0.20	0.48 ± 1.01	3.28 ± 0.55
	III	9	0.26 ± 0.20	0.50 ± 0.34	1.32 ± 0.57

[1] Values are the means ± SEM

[2] Values are the mean levels after the discontinuation of TPN; or if the infant received a CNE-containing formula prior to the discontinuation of TPN, the values is the mean plasma level prior to the provision of the CNE-containing formula

[3] Non-esterified carnitine (nmol/ml)

[4] Acid-soluble acylcarnitine (nmol/ml)

[5] Acid-insoluble acylcarnitine (nmol/ml)

[6] Total carnitine (nmol/ml)

[7] Triglyceride (mg/dl)

[8] Fat intake (g/kg/day)

[a] pre-TPN and post-TPN values are different a $p<0.05$ level

significant decrease of ASAC was seen only in Group III. The NEC:ASAC ratio increased in Groups I and III, while at the same time it decreased in Group II (Table A-5)

The levels of NEC and TC decreased in Groups I and II throughout TPN administration. However, in Group III, the levels of NEC, ASAC, and TC were increased from the 24 hour post-TPN levels, but were still below pre-TPN levels. Upon discontinuation of TPN (Post-TPN), the TC levels were 8.2, 14.5, and 18.2 nmol/ml for Groups I, II, and III, respectively. These values compared with the pre-TPN values of 36.3, 25.4, and 22.8 nmol/ml and the 24 hour post-TPN values of 24.1, 21.6, and 16.3 nmol/ml for each of the respective groups. The mean numbers of days that each group received TPN (before administration of a carnitine-containing formula) were 35.0, 13.5, and 5.5, respectively.

The only significant change in the plasma TG level at 24 hours post-TPN occurred in Group II where there was a decrease of TG. There were no significant changes in plasma triglyceride upon discontinuation of TPN.

2. Carnitine and Triglyceride Changes After Intravenous Fat Emulsion Administration

The changes in carnitine and TG levels after administration of intravenous fat emulsion are presented in Table 7 and Figure A-2. There were no significant changes in any of the carnitine fractions 24 hours after continuous infusion of intravenous fat emulsion when compared to pre-fat infusion values. However, all groups showed an increase in the mean concentrations of NEC and TC. Only Group III had an increase in the mean ASAC level, while the other two groups showed

Table 7. Plasma Carnitine and Triglyceride Levels[1] Before and 24 Hours After Initiation of Intravenous Fat Emulsion.

Parameter	Group	n	Pre-Fat	Post-Fat
NEC[2]	I	8	18.7 ± 3.3	19.8 ± 2.7
	II	6	15.0 ± 2.0	19.2 ± 3.0
	III	3	11.0 ± 2.7	12.1 ± 3.4
ASAC[3]	I	8	6.4 ± 2.1	4.7 ± 2.7
	II	6	5.9 ± 2.3	3.7 ± 0.6
	III	3	2.4 ± 1.0	3.3 ± 0.5
AIAC[4]	I	8	1.2 ± 0.3	1.2 ± 0.2
	II	6	0.7 ± 0.1	0.7 ± 0.2
	III	3	0.8 ± 0.2	0.6 ± 0.1
TC[5]	I	8	26.7 ± 4.6	28.8 ± 5.0
	II	6	21.6 ± 2.3	23.7 ± 3.3
	III	3	14.1 ± 3.9	17.8 ± 2.2
TG[6]	I	8	56.2 ± 10.0	75.9 ± 10.9 [a]
	II	6	43.2 ± 6.4	46.5 ± 11.0
	III	3	44.7 ± 4.8	46.0 ± 10.5
Fat Intake[7]	I	8	0.00 ±	0.32 ± 0.07
	II	6	0.18 ± 0.18	0.32 ± 0.17
	III	3	0.00 ±	0.24 ± 0.13

[1] Values are the means ± SEM
[2] Non-esterified carnitine (nmol/ml)
[3] Acid-soluble acylcarnitine (nmol/ml)
[4] Acid-insoluble acylcarnitine (nmol/ml)
[5] Total carnitine (nmol/ml)
[6] Triglyceride (mg/dl)
[7] Fat intake (g/kg/day)

[a] pre-fat and post-fat values are different at $p<0.05$ level

virtually no change in ASAC. The mean NEC:ASAC ratio decreased in both Groups I and III, but increased in Group II (Table A-5). There were no significant changes of AIAC in any group when compared to the pre-administration levels.

The plasma TG concentration remained constant in both Groups II and III during the 24 hours of fat administration. Only Group I had a significant increase in plasma triglyceride with administration of intravenous fat emulsion. However, Group I also had the largest increase in the mean total fat intake than either of the other groups.

3. Changes of Carnitine and Triglyceride After Portagen Feedings

The carnitine and TG changes before and after achievement of full strength Portagen are presented in Table 8 and Figure A-3. The NEC and TC levels decreased in Groups I and II, but had not changed in Group III when compared to the pre-Portagen plasma levels. The NEC:ASAC ratio decreased in all groups during the administration of Portagen (Table A-5). There were no significant changes of ASAC, AIAC, or TG at 24 hours after full strength Portagen feedings when compared to the pre-Portagen values.

4. Changes of Carnitine and Triglyceride After Enfamil-Premature Formula Feedings

The carnitine and TG levels before and at 24 hours after achievement of full strength Enfamil-Premature Formula (Enfamil-PF) are presented in Table 9 and Figure A-4. These values reflect only infants in Groups I and II, as no infant in Group III received Enfamil-PF. The mean levels of TC, NEC, and TG were increased in both groups when

Table 8. Plasma Carnitine and Triglyceride Levels[1] Before and After Initiation of Full Strength Portagen.

Parameter	Group	n	Pre-Portagen	Post-Portagen
NEC[2]	I	5	17.9 ± 3.1	5.1 ± 0.9 [a]
	II	8	17.7 ± 2.3	10.5 ± 1.9 [a]
	III	2	13.4 ± 1.0	10.9 ± 0.2
ASAC[3]	I	5	4.7 ± 0.9	2.7 ± 0.5
	II	8	3.6 ± 0.4	4.3 ± 0.7
	III	2	2.2 ± 0.7	3.4 ± 0.7
AIAC[4]	I	5	0.9 ± 0.2	0.6 ± 0.2
	II	8	0.8 ± 0.2	0.5 ± 0.1
	III	2	0.9 ± 0.2	0.9 ± 0.2
TC[5]	I	5	23.5 ± 3.6	8.5 ± 1.4 [a]
	II	8	21.6 ± 2.1	15.3 ± 2.5 [a]
	III	2	18.7 ± 1.8	15.2 ± 1.2
TG[6]	I	5	63.4 ± 8.9	58.4 ± 3.1
	II	8	61.9 ± 11.0	87.2 ± 17.3
	III	2	44.5 ± 4.0	113.4 ± 64.8
Fat Intake[7]	I	5	0.37 ± 0.13	4.16 ± 0.34
	II	8	0.54 ± 0.16	3.85 ± 0.16
	III	2	0.00 ±	1.08 ± 1.11

[1] Values are the means ± SEM
[2] Non-esterified carnitine (nmol/ml)
[3] Acid-soluble acylcarnitine (nmol/ml)
[4] Acid-insoluble acylcarnitine (nmol/ml)
[5] Total carnitine (nmol/ml)
[6] Triglyceride (mg/dl)
[7] Fat intake (g/kg/day)

[a] pre-Portagen and post-Portagen values are different at $p<0.05$ level

Table 9. Plasma Carnitines and Triglyceride Levels[1] Before and 24 Hours After Initiation of Full Strength Enfamil-Premature Formula.

Parameter	Group	n	Pre-Enfamil-PF	Post-Enfamil-PF
NEC[2]	I	3	5.4 ± 1.0	18.0 ± 4.7 [a]
	II	2	10.0 ± 5.8	22.7 ± 6.5
	III	0		
ASAC[3]	I	3	0.9 ± 0.6	4.7 ± 1.8
	II	2	5.4 ± 1.5	5.5 ± 1.2
	III	0		
AIAC[4]	I	3	0.3 ± 0.1	0.6 ± 0.2
	II	2	1.1 ± 0.7	0.5 ± 0.5
	III	0		
TC[5]	I	3	6.5 ± 1.6	22.4 ± 8.1 [a]
	II	2	16.5 ± 8.0	28.8 ± 8.5
	III	0		
TG[6]	I	3	93.0 ± 14.6	102.7 ± 32.7
	II	2	56.0 ± 12.0	89.0 ± 42.0 [a]
	III	0		
Fat Intake[7]	I	3	3.75 ± 0.52	5.23 ± 0.17
	II	2	1.95 ± 1.14	4.71 ± 0.62
	III	0		

[1] Values are the means ± SEM
[2] Non-esterified carnitine (nmol/ml)
[3] Acid-soluble acylcarnitine (nmol/ml)
[4] Acid-insoluble acylcarnitine (nmol/ml)
[5] Total carnitine (nmol/ml)
[6] Triglyceride (mg/dl)
[7] Fat intake (g/kg/day)

[a] pre-Enfamil-PF and post-Enfamil-PF values are different at $p<0.05$ level

compared to the pre-Enfamil-PF levels. The NEC:ASAC ratio decreased in Group I while at the same time, the ratio increased in Group II. The levels of ASAC and AIAC had not significantly changed in either group when compared to the pre-Enfamil-PF plasma levels.

5. Changes of Carnitine and Triglyceride After Enfamil-20 feedings

This classification consists only of infants which received Enfamil-20 as their first carnitine-containing formula. All infants who had received Enfamil-PF or breastmilk prior to Enfamil-20 feedings were excluded. The carnitine and TG changes after Enfamil-20 feedings are presented in Table 10 and Figure A-5. The concentrations of NEC, ASAC, AIAC, and TC were increased 24 hours after achievement of full strength Enfamil-20. The mean NEC, ASAC, TC, and TG levels were increased in both groups when compared to pre-Enfamil-PF levels. The mean AIAC level was increased in Groups I and III, while at the same time it remained constant in Group II. The NEC:ASAC ratio was increased in both Groups I and II, but was decreased in Group III when compared to pre-Enfamil-20 values (Table A-5). The TG level was wignificantly higher in Group III, while no significant changes were seen in either Group I or Group II in the post-Enfamil-20 sampling.

6. Changes of Carnitine and Triglyceride After Breastmilk Feedings

Plasma carnitine and TG levels before and after breastmilk feedings are presented in Table 11 and Figure A-6. The values include infants from Groups II and III only, as there were no infants in Group I who received breastmilk in the study. The levels of NEC, ASAC, AIAC, TC, and TG all increased from the pre-breastmilk values.

Table 10. Plasma Carnitine and Triglyceride Levels[1] Before and 24 Hours After the Initiation of Full Strength Enfamil-20.

Parameter	Group	n	Pre-Enfamil-20	Post-Enfamil-20
NEC[2]	I	3	3.0 ± 0.8	12.0 ± 1.0 [a]
	II	6	7.9 ± 1.6	23.1 ± 3.9 [a]
	III	14	12.6 ± 1.1	18.6 ± 1.3 [a]
ASAC[3]	I	3	1.3 ± 0.2	4.0 ± 1.1
	II	6	3.8 ± 0.6	6.9 ± 1.0 [a]
	III	14	4.2 ± 0.5	8.1 ± 0.9 [a]
AIAC[4]	I	3	0.1 ± 0.0	0.3 ± 0.0
	II	6	0.5 ± 0.2	0.5 ± 0.1
	III	14	0.9 ± 0.2	1.0 ± 0.1
TC[5]	I	3	4.4 ± 0.5	15.3 ± 2.1 [a]
	II	6	12.3 ± 2.2	32.6 ± 4.8 [a]
	III	14	17.7 ± 1.3	27.7 ± 1.9 [a]
TG[6]	I	3	52.7 ± 1.5	55.0 ± 8.7
	II	6	72.9 ± 20.5	116.2 ± 44.2
	III	14	39.0 ± 8.4	102.5 ± 13.4 [a]
Fat Intake[7]	I	3		
	II	6	4.44 ± 0.25	6.00 ± 0.21
	III	14	0.04 ± 0.03	4.31 ± 0.34

[1] Values are the means ± SEM
[2] Non-esterified carnitine (nmol/ml)
[3] Acid-soluble acylcarnitine (nmol/ml)
[4] Acid-insoluble acylcarnitine (nmol/ml)
[5] Total carnitine (nmol/ml)
[6] Triglyceride (mg/dl)
[7] Fat intake (g/kg/day)

[a] pre-Enfamil-20 and post-Enfamil-20 values are different at $p<0.05$ level

Table 11. Plasma Carnitine and Triglyceride Levels[1] Before and 24 Hours After the Initiation of Full Strength Breastmilk.

Parameter	Group	n	Pre-Breastmilk	Post-Breastmilk
NEC[2]	I	0		
	II	3	14.2 ± 1.5	23.8 ± 1.6 [a]
	III	5	20.1 ± 2.0	24.2 ± 3.2
ASAC[3]	I	0		
	II	3	3.5 ± 0.8	6.7 ± 1.6
	III	5	6.4 ± 1.1	7.9 ± 1.4
AIAC[4]	I	0		
	II	3	0.8 ± 0.2	1.3 ± 0.1
	III	5	1.1 ± 0.2	1.5 ± 0.1
TC[5]	I	0		
	II	3	18.5 ± 1.1	31.8 ± 1.8 [a]
	III	5	27.5 ± 3.0	33.7 ± 4.5 [a]
TG[6]	I	0		
	II	3	43.6 ± 13.4	80.0 ± 33.2
	III	5	80.5 ± 22.7	94.8 ± 12.4
Fat Intake[7]	I	0		
	II	3	2.03 ± 1.21	3.99 ± 0.30
	III	5	2.76 ± 0.69	4.43 ± 0.19

[1] Values are the means ± SEM
[2] Non-esterified carnitine (nmol/ml)
[3] Acid-soluble acylcarnitine (nmol/ml)
[4] Acid-insoluble acylcarnitine (nmol/ml)
[5] Total carnitine (nmol/ml)
[6] Triglyceride (mg/dl)
[7] Fat intake (g/kg/day)

[a] pre-breastmilk and post-breastmilk values are different at $p<0.05$ level

The NEC:ASAC ratio was virtually unchanged in both groups (Table A-5). The increase in TG levels paralleled the increase of fat intake, but the changes were were not significantly different from pre-breastmilk levels in either group.

E. Plasma Carnitine Changes With Carnitine Intake

The relationship between plasma carnitine levels and dietary carnitine intake is presented in Table 12 and Figures 7-10. Both carnitine intake reported in µmol/kg/day and in total µmol/day were correlated with an increase in the plasma TC, NEC, and ASAC levels. However in all groups, the plasma NEC and CNE levels were slightly better correlated with carnitine intake when the intake was based on µmol/day rather than µmol/kg/day. The difference between the two expressions (umol/day or umol/kg/day) was highest in the smallest infants (Group I), followed by Group II, while the difference had almost disappeared in Group III. The plasma ASAC and AIAC were not consistently better correlated with carnitine intake expressed either in µmol/day or µmol/kg/day. All groups responded to an increased intake of dietary carnitine with an increase of plasma TC, NEC, and ASAC. The slope of the regression line of TC, NEC, and ASAC was higher in Group I infants, however, this group also had the lowest carnitne levels prior to carnitine intake. Unlike the other groups, infants in Group I showed an increase of AIAC with carnitine intake. Between the infants of Groups II and III, both showed similar increases in plasma TC, NEC, and ASAC levels for any given carnitine intake.

Table 12. Correlations Between Plasma Carnitine Concentrations and Daily Carnitine Intake.

Parameter	Group	r	p	Regression Line
Carnitine Intake (μmol/kg/day)				
TC	I	0.779	1.06E-06	Y = 0.859*X + 8.913
	II	0.791	2.09E-14	Y = 0.886*X + 18.238
	III	0.707	<1.00E-15	Y = 0.857*X + 17.928
NEC	I	0.735	8.19E-06	Y = 0.656*X + 7.247
	II	0.808	2.22E-05	Y = 0.805*X + 11.825
	III	0.696	3.69E-07	Y = 0.696*X + 12.034
ASAC	I	0.752	3.86E-06	Y = 0.189*X + 1.405
	II	0.384	2.03E-03	Y = 0.093*X + 5.438
	III	0.575	6.84E-08	Y = 0.170*X + 4.643
AIAC	I	0.515	5.08E-03	Y = 0.152*X + 0.210
	II	-0.422	6.34E-04	Y = -0.013*X + 0.978
	III	-0.197	4.20E-02	Y = -0.009*X + 1.252
Carnitine Intake (μmol/day)				
TC	I	0.882	5.83E-10	Y = 0.596*X + 4.410
	II	0.841	<1.00E-15	Y = 0.431*X + 19.446
	III	0.721	<1.00E-15	Y = 0.436*X + 18.311
NEC	I	0.851	9.72E-09	Y = 0.465*X + 3.335
	II	0.882	<1.00E-15	Y = 0.402*X + 12.528
	III	0.715	<1.00E-15	Y = 0.356*X + 12.280
ASAC	I	0.787	6.78E-07	Y = 0.121*X + 0.889
	II	0.317	1.20E-02	Y = 0.035*X + 5.956
	III	0.567	4.39E-08	Y = 0.084*X + 4.808
AIAC	I	0.582	1.16E-03	Y = 0.011*X + 0.130
	II	-0.453	2.15E-04	Y = -0.006*X + 0.963
	III	-0.167	8.50E-02	Y = -0.004*X + 1.226

Variables defined in text.
Y=dependent variable (plasma carnitine)
X=independent variable (carnitine intake)

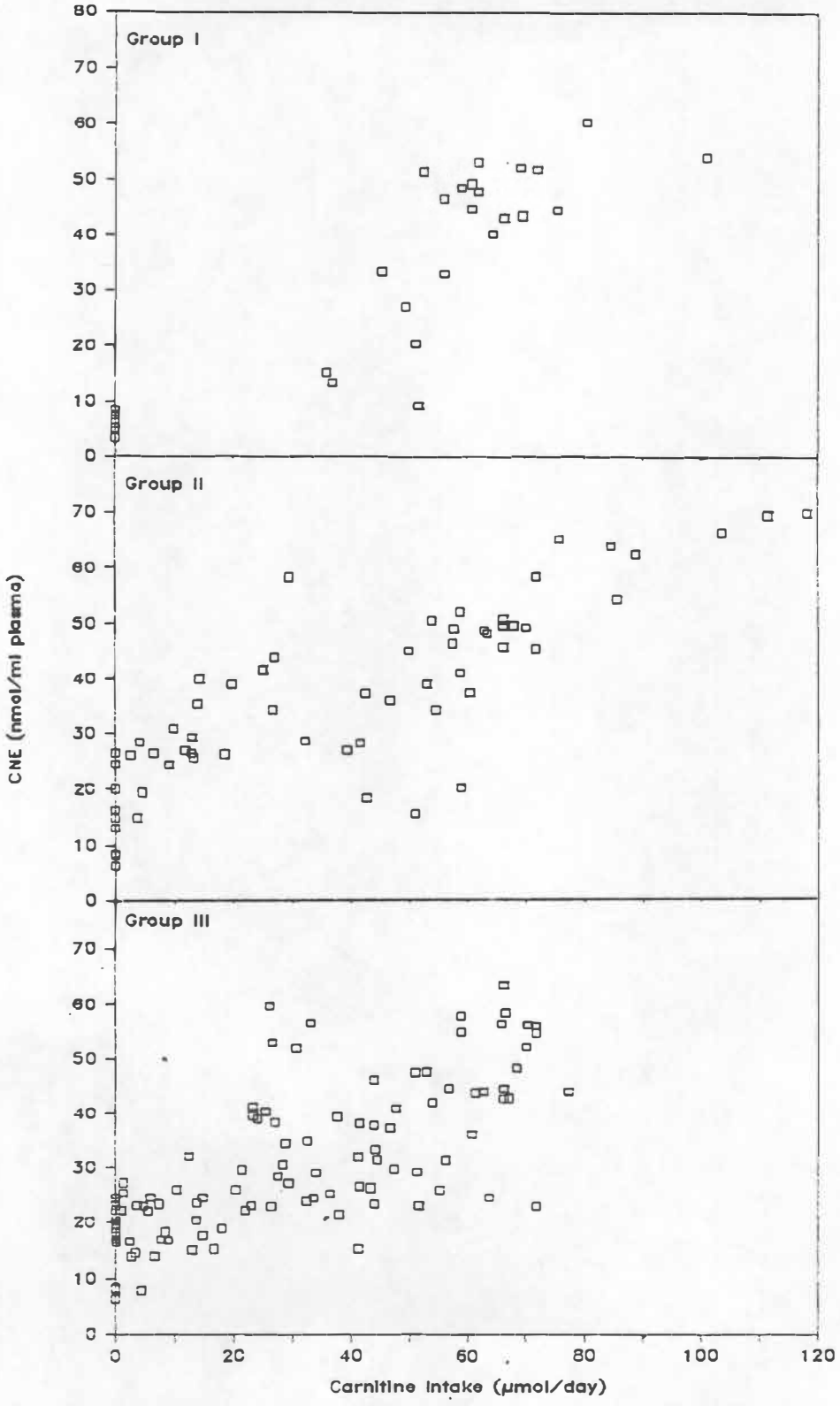

Figure 7. Relationship between Plasma Total Carnitine Concentration and Daily Carnitine Intake.

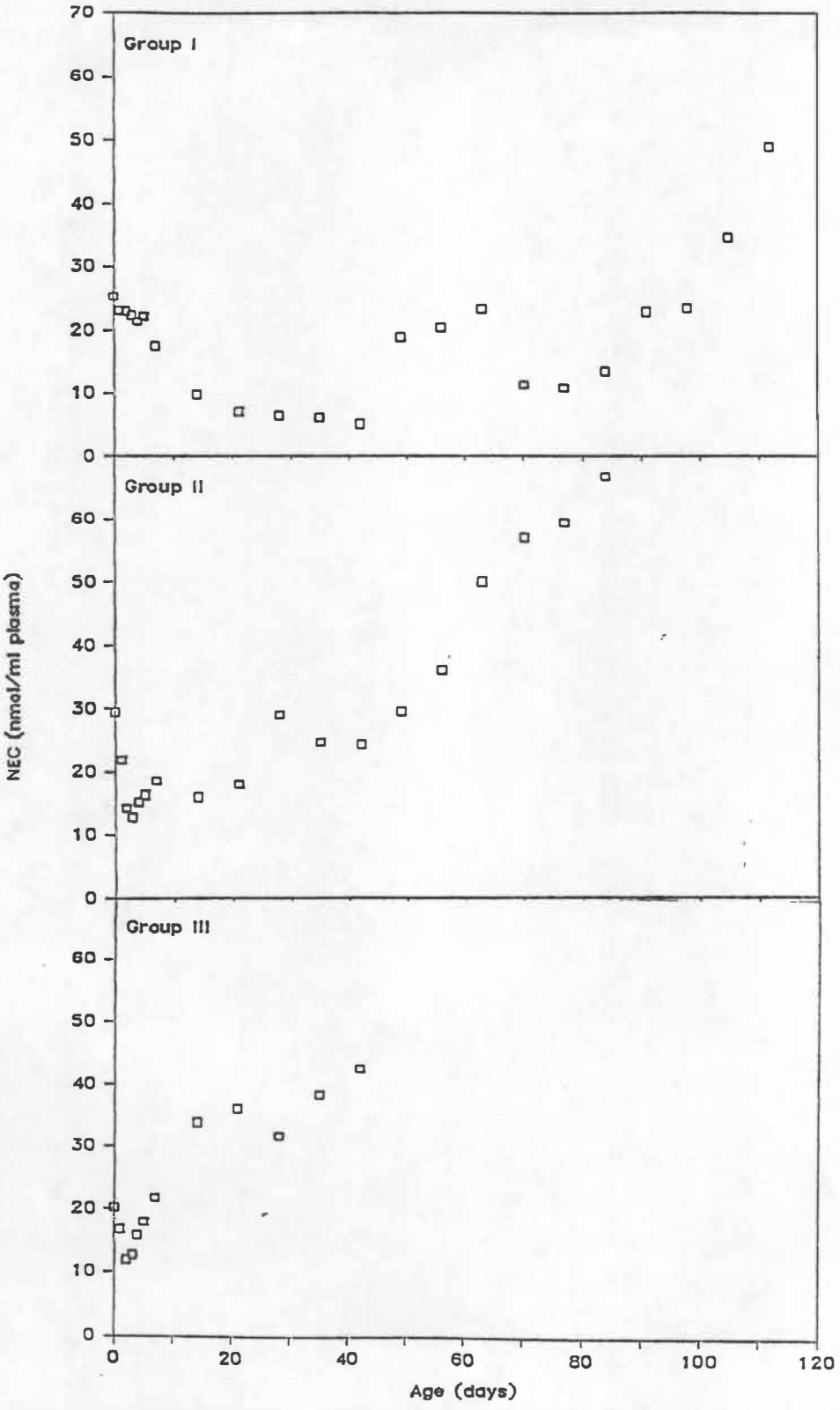

Figure 8. Relationship Between Plasma NEC Concentration and Daily Carnitine Intake.

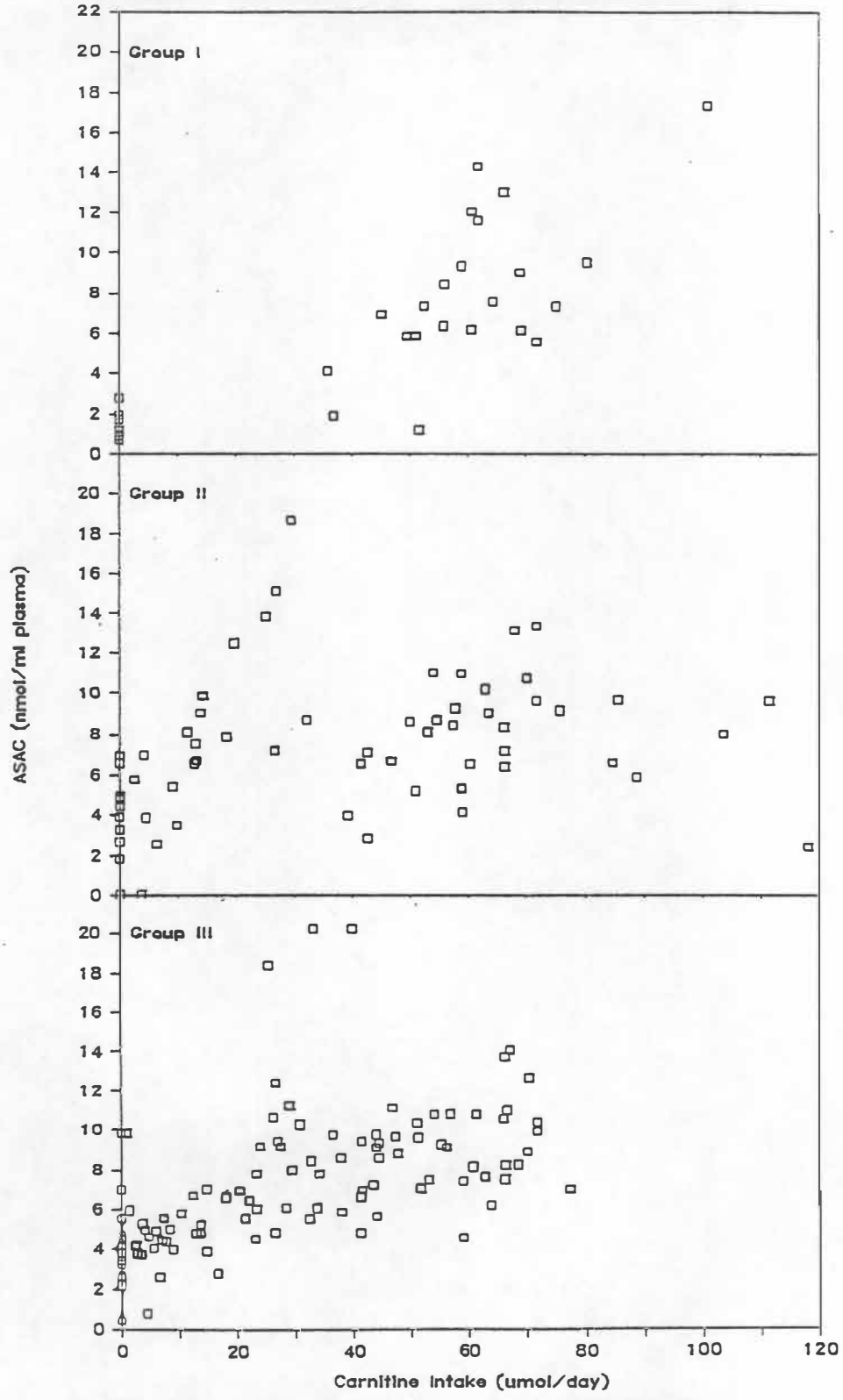

Figure 9. Relationship between Plasma ASAC Concentration and Daily Carnitine Intake.

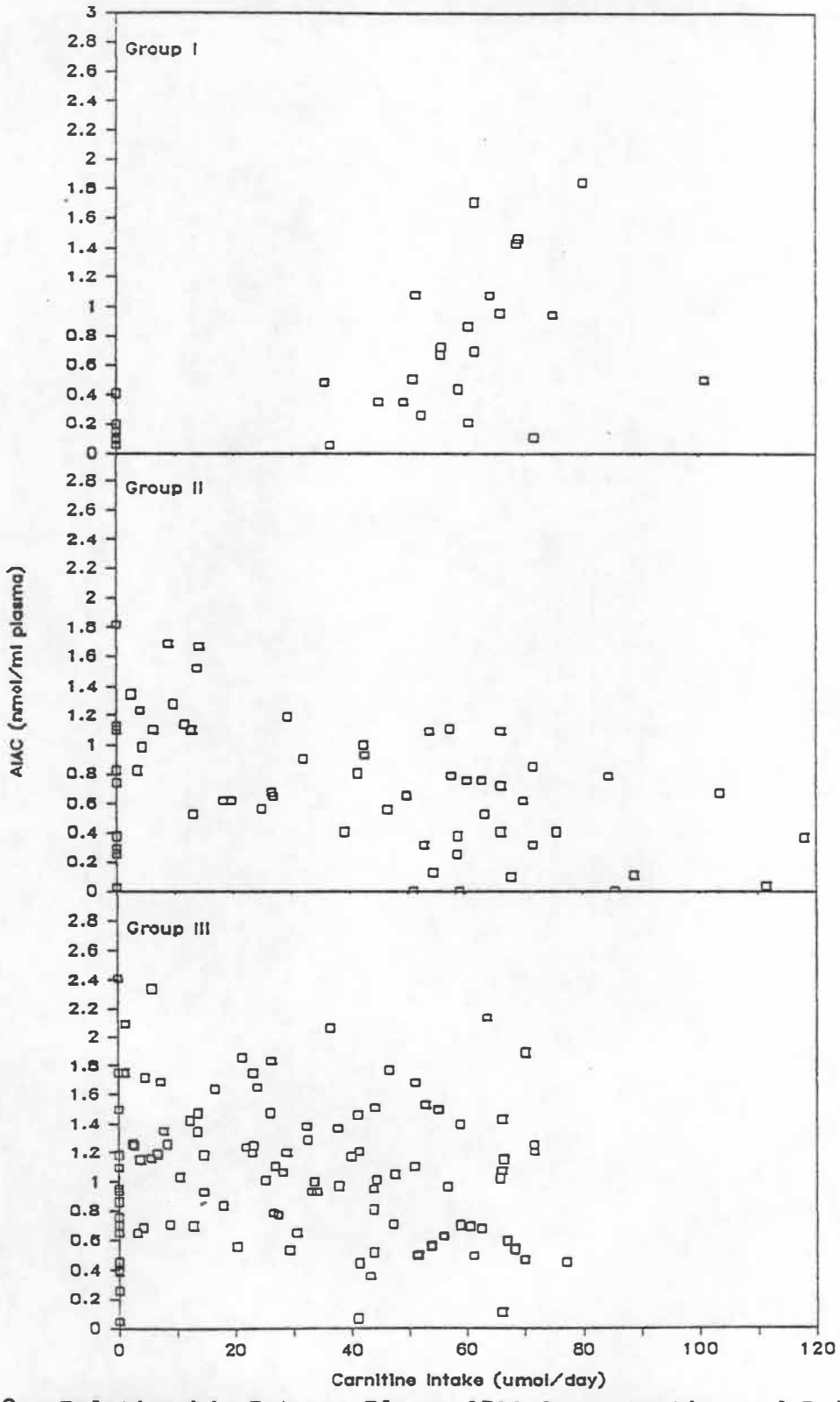

Figure 10. Relationship Between Plasma AIAC Concentration and Daily Carnitine Intake.

F. Relationship of Plasma Carnitine and Lipids

1. Relationship of Plasma Carnitine and Triglyceride

The correlations between carnitine and TG levels of infants in Groups I-III are presented in Table 13. There were no significant correlations in Group I between any of the plasma carnitines and TG levels. In both Groups II and III, there were significant positive correlations between triglyceride level and the levels of NEC ($r=0.211$, $p<0.01$ and $r=0.337$, $p<0.0001$), ASAC ($r=0.308$, $p<0.001$ and $r=0.409$, $p<0.0001$), and TC ($r=0.255$, $p<0.01$ and $r=0.370$, $p<0.0001$) for Groups II and III, respectively. The mean NEC:ASAC ratio was not significantly correlated with triglyceride level in any group (Table A-6), and there were no significant correlations between AIAC and triglyceride levels (Table A-7) in any group.

2. Relationship of Plasma Carnitine and Non-esterified Fatty Acids

Non-esterified fatty acids were only measured on a limited number ($n=44$) of plasma samples of infants from the most premature infants (Group I, $n=144$ total plasma samples). From these limited data, a positive correlation was found between plasma TC and NEFA levels ($r=0.381$, $p<0.05$) as shown in Figure 11 and between ASAC and NEFA levels ($r=0.344$, $p<0.025$) as shown in Figure 12.

G. Relationship of Short-chain Acylcarnitines and Fat Intake

The correlations between short-chain acylcarnitines (ASAC) and fat intake are presented in Table 14. There was a positive correlation between fat intake and ASAC in Groups II and III. The correlation

Table 13. Correlation Between Plasma Carnitine and Triglyceride Concentrations of Individual Infants by Group

Parameter	Group	r	p
NEC	I	-0.098	NS
	II	0.211	<0.01
	III	0.337	<0.0001
ASAC	I	-0.034	NS
	II	0.308	<0.001
	III	0.409	<0.0001
AIAC	I	0.004	NS
	II	-0.101	NS
	III	-0.127	NS
TC	I	-0.084	NS
	II	0.225	<0.01
	III	0.370	<0.0001

Variables defined in text.

Table 14. Correlation Between Plasma Acid-Soluble Acylcarnitine Concentration and Fat Intake.

Group	r	p	Regression Line
I	-0.152	NS	
II	0.400	<0.0001	Y = 0.546*X + 4.127
III	0.614	<0.0001	Y = 0.809*x + 4.230

Variables defined in text.

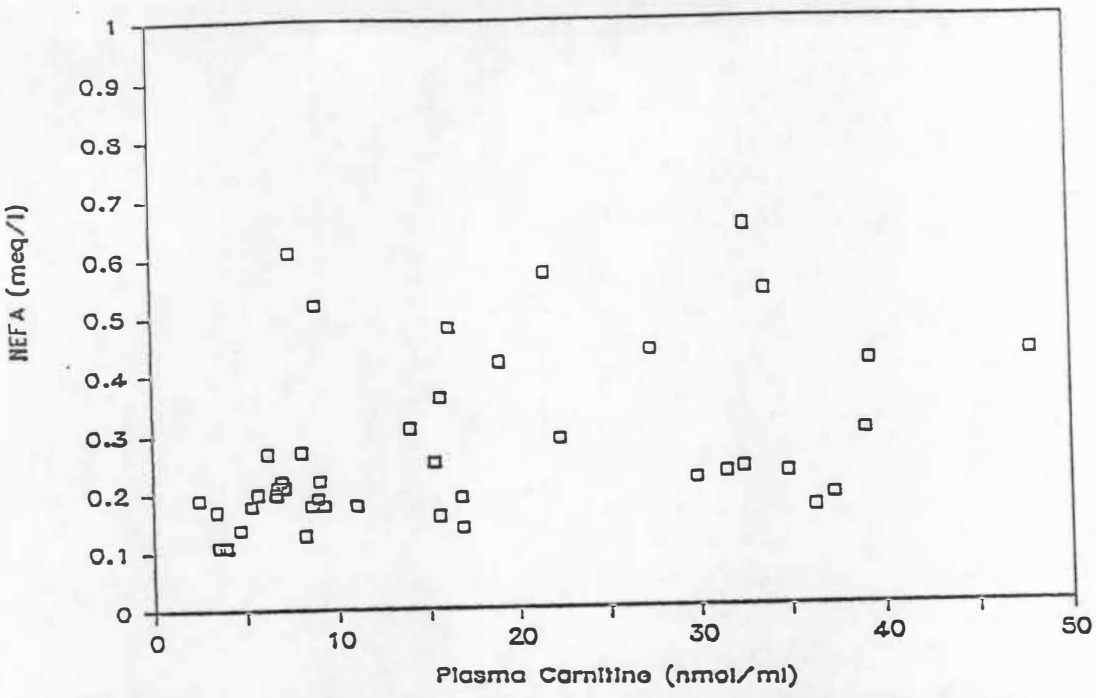

Figure 11. Relationship Between Plasma Non-esterified Fatty Acid and Total Carnitine Concentrations of a Random Sample of Group I Infants.

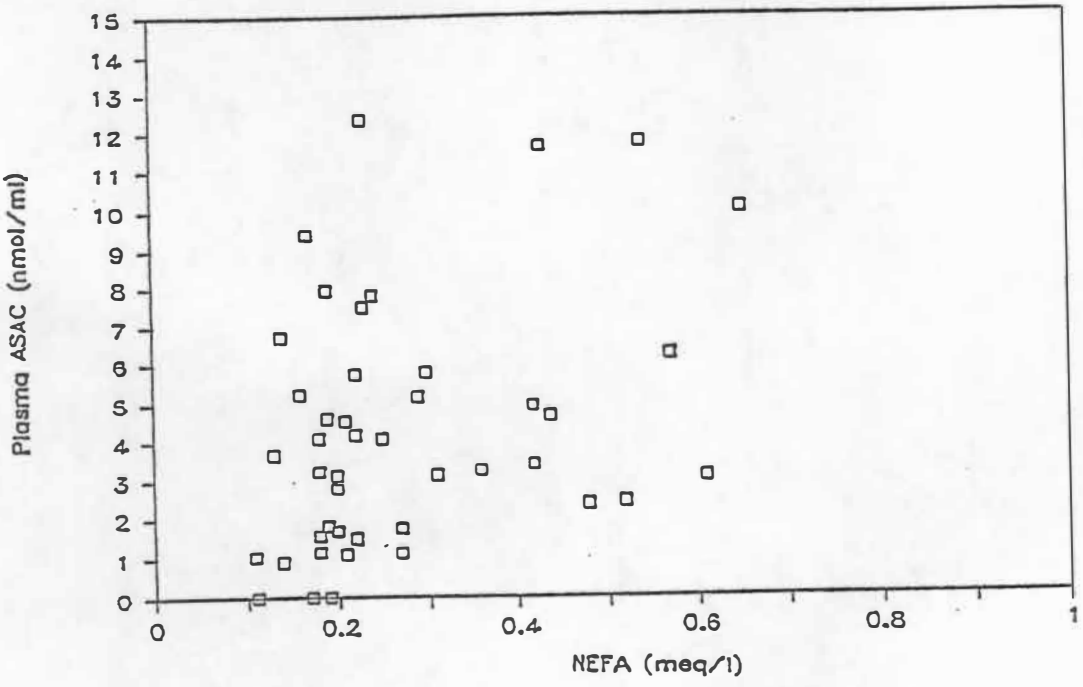

Figure 12. Relationship Between Plasma ASAC and Non-esterified Fatty Acid Concentrations in a Random Sample of Group I Infants.

coefficients were r=0.400 (p<0.0001), and r=0.614 (p<0.0001) for Groups II and III respectively. There was no significant correlation between ASAC and fat intake in Group I (r=-0.152, p>0.05).

H. Plasma Lipids Related to Fat Intake

1. Plasma Triglyceride Related to Fat Intake

The correlation between plasma triglyceride and fat intake is presented in Table 15. The plasma triglyceride concentration was not correlated to fat intake (g/kg/day) in Group I. However, there was a significant positive correlation between triglyceride and fat intake in Groups II and III (r=0.539, p<0.001; and r=0.634, p<0.0001, respectively).

2. Plasma Non-esterified Fatty Acids Related to Fat Intake

As mentioned above, NEFA were determined on a limited sample of Group I infant plasma. Of these, no correlation was found between the plasma NEFA level and fat intake (r=-0.086, p=0.581).

I. Plasma Triglyceride Related to Calorie Intake

The correlations between plasma TG level and calorie intake (kcal/kg/day) are presented in Figure 13. The TG level demonstrated a significantly positive correlation with calorie intake. The correlation was highest in Group III (r=0.60, p<0.00001), followed by Group II (r=0.473, p<0.0001), and Group I (r=0.311, p<0.001).

Table 15. Correlation Between Plasma Triglyceride Concentration and Fat Intake.

Group	r	p	Regression Line
I	0.194	NS	
II	0.539	<0.001	Y = 9.816*X + 41.512
III	0.634	<0.00001	Y = 12.754*X + 38.289

Variables defined in text.

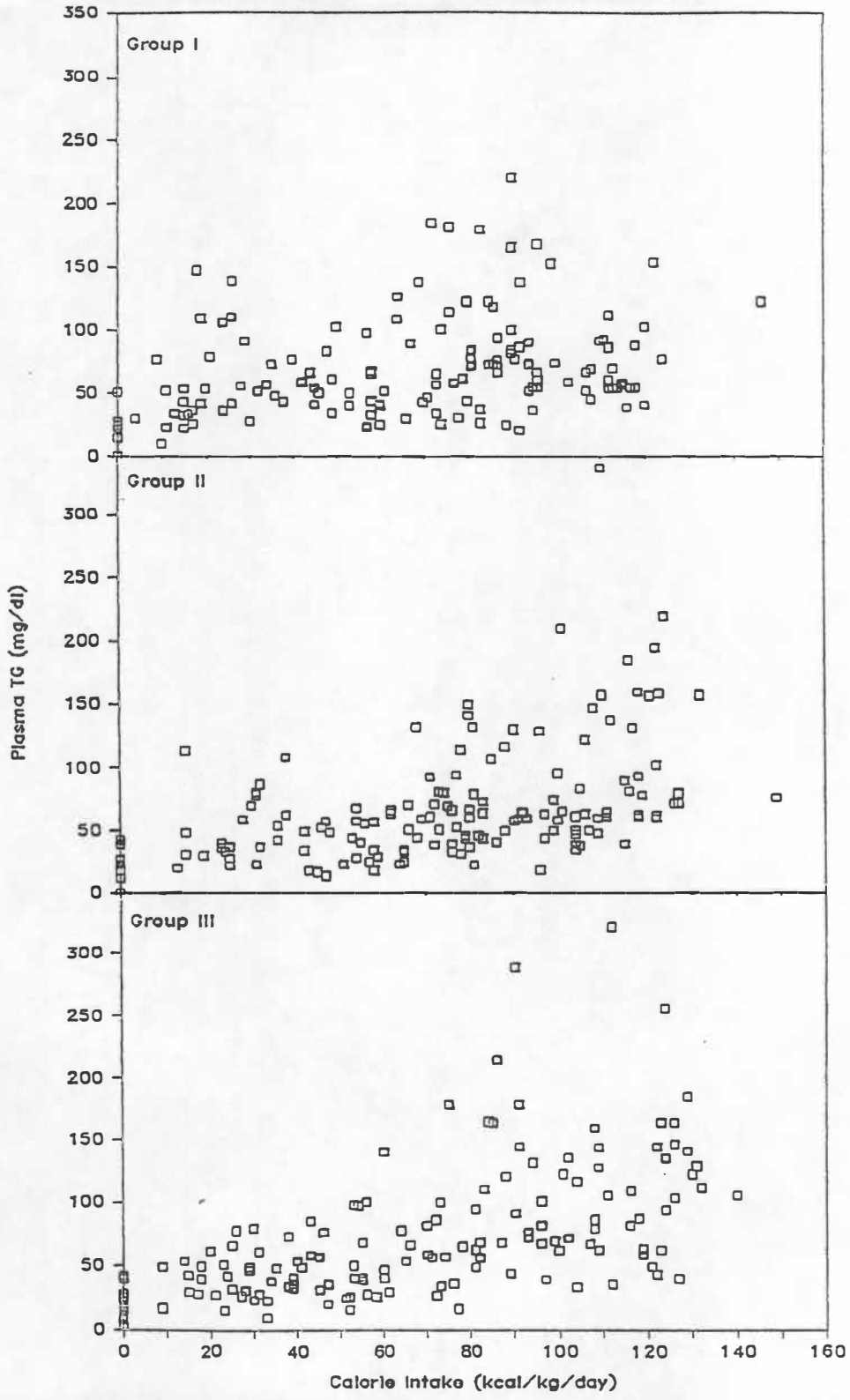

Figure 13. Relationship Between Plasma Triglyceride Concentration and Daily Calorie Intake.

CHAPTER V

DISCUSSION

Since the concentration of plasma carnitine in infants is dependent upon a dietary source of carnitine, rather than endogenous biosynthesis, it is conceivable that infants who receive a carnitine-free diet may have an altered lipid metabolism. No study has been done with multiple subjects for an extended period of time in which the carnitine and lipid status of infants have been monitored during various nutritional interventions. To acheive the objective of the study, the following paramenters were investigated: (1) birth (baseline) concentrations of carnitine in the plasma of the infant, umbilical cord, and mother at various gestational stages; (2) plasma carnitine levels following various infant feedings; and, (3) changes in plasma carnitine in relation to the changes in the plasma lipid concentrations.

The infants born at <1510 g (Groups I and II) had a higher plasma carnitine level than either the older premature or the term infants at birth which is in agreement with other studies (57). The carnitine levels in infants were higher than either the maternal or umbilical cord plasma levels at birth (Figure 1 and Table 3, pp. 39-40). In general, the plasma carnitine levels in infants decreased with advancing gestational age after birth, which is in concert with the observations of Shenai and Borum (71). However, the 24.5 to 31 weeks infants exhibited a sharp rise in carnitine which was positively correlated with advancing gestational age during this period. Infants

of 31.5 to 34 weeks gestation age showed a sharp decline in plasma carintine during this period of development. The infants of gestational age >34 weeks maintained the plasma levels of NEC and TC at about 15 and 20 nmol/ml, respectively (Figures 2 and 3, pp. 44-45). These data suggest that the infant acquired carnitine from the maternal blood supply which resulted in accumulation of carnitine in the plasma pool of the infant during the weeks 24-31 of gestation. The plasma pool appeared to decrease during weeks 31-35 of gestation which may have been due to transfer of plasma carnitine into fetal tissues. It has been suggested that carnitine receptors become active with advancing stages of gestation (47,71). This suggestion is based upon the presence of, (1) a cardiac carnitine binding protein in ventricular homogenates of humans (89) and in the isolated rat myocardium (90), and (2) active transport mechanisms described in human skeletal muscle (91) and liver (92) whose maturation time in fetal development is not known.

Levels of NEC and TC were decreased in all infants 2 days after birth unless they were provided with a source of dietary carnitine. However, on day 0 and day 1, there were marked differences in the percent changes of carnitine levels between groups. In Group I, the levels of TC and ASAC increased, while at the same time NEC was decreased compared with birth values. This may reflect release of free carnitine from muscle with subsequent transport to hepatic tissues, an effect mediated by an increased level of glucagon following birth (93). In addition, the high levels of plasma TC may

be due to decreased renal excretion of carnitine since premature infant kidneys are not fully functional.

During this same period (days 0 and 1), Groups II and III showed a decrease in both NEC and TC. Group II had a greater decrease in NEC and TC than Group III. A possible explanation may be that the renal tubular threshold was exceeded, or exceeded to a higher degree in Group II due to their higher plasma levels of carnitine. The renal threshold of carnitine has not been determined in infants. In adults, the renal plasma threshold for carnitine excretion was 46.71 and 51.74 nmol/ml for NEC and TC, respectively (94). It is conceiveable that infants may have a lower renal threshold for carnitine when compared to adults. Therefore, as opposed to Group I infants who were unable to excrete carnitine, the older infants in Groups II and III may have excreted carnitine based on their plasma carnitine level. Since Group II had a much higher plasma carnitine that Group III at birth, a higher renal excretion may have accounted for their marked decline in plasma carnitine after the maternal-placental supply of carnitine no longer existed.

Beginning at day 2, some infants in Group III began to receive a carnitine-containing formula, and on day 4, some of the Group II infants also began to receive a carnitine-containing formula. After this period of the study, the groups could not be meaningfully compared with one another in terms of absolute mean values of carnitine with time (age), but rather only group trends could be observed due to the different feeding regimes within and between each group.

The NEC:ASAC ratio remained meaningful since this ratio reflects a metabolic state, whereas the absolute values of each tend to reflect intake (or lack of intake) of dietary carnitine. A decrease in this ratio is correlated with the ability of the infant to oxidize NEFA and to undergo ketogenesis for the subsequent release of metabolic energy (23). Since the NEC:ASAC ratio decreased in all groups during the post-partum period, it appeared that they were able to oxidize lipid to some degree. However the absolute values of the ketone bodies and NEFA will be needed in future studies to qualify and quantify the significance of the NEC:ASAC ratio. Yeh et al. (76) found that premature infants were only able to attain 40-45% of the ketone body levels seen in term infants when NEFA were equally available.

The NEC:ASAC ratio did not increase to the birth values until the calorie intake was approximately 100-110 kcal/kg/day. This may have indicated that the infant depends on endogenous and exogenous fat as an energy source until reaching the stated energy intake. Once a higher calorie intake is achieved there would be little need for NEFA mobilization and ketogensis to support the energy needs of the infant.

The AIAC levels increased in all infants after birth (Figure 5, p. 46). Since there is a high glucagon to insulin ratio durng the first day after birth (93), the theory of McGarry et al. (93) may explain the increased AIAC level. Muscle carnitine may be transported to the liver to stimulate carnitine palmitoytransferase (CPT) activity with a subsequent increase in long-chain acylcarnitine level. This would be reflected by an increase level of AIAC in the plasma. In other studies, the group of McGarry and others (95,96), proposed that

increased glucagon (an increased glucagon to insulin ratio) not only stimulates the concentration of carnitine in the liver, but a low hepatic carnitine level may stimulate glucagon levels in the body to overcome a lack of carnitine in the liver. The level of hepatic carnitine has been found to be lower in the neonate than in the adult, however, there was no difference between premature and term infants (71).

The theory of McGarry and co-workers may also explain why Group I exhibited the highest levels of ASAC and the lowest ratio of NEC:ASAC during the first week of life. Since Group I maintained peak AIAC for a longer period of time than any other group, this may reflect a continued stimulation of hepatic CPT or just a continued higher degree of long-chain fatty acid oxidation. This would increase the level of acetylcarnitine, the primary short-chain acylcarnitine (ASAC) in the plasma.

Another intent of this study was to observe plasma TG levels when the plasma carnitine concentration was depressed. As seen in Table 13 (p. 68) a depressed plasma carnitine concentration was not correlated with an elevated TG level. In Group I, the mean plasma TC level decreased to its lowest level of 6.8 nmol/ml while the mean TG level was only 69 mg/dl. The highest mean TG seen in this group was 153 mg/dl on day 105, while the CNE level was 47.8 nmol/ml. This unexpected observation may be attributed to the small amount of long-chain TG provided to these infants. Although most infants were placed on TPN with simultaneous intravenous fat emulsion, the typical lipid dose administered was less than 1-1.5 g/kg/day (calculated from

individual infants during the administration of intravenous lipid fat emulsion). The highest isolated dose given to an individual infant in Group I was 2.14 g/kg/day followed by an isolated dose of 1.84 g/kg/day. The plasma TG which coincided with these fat intakes were 99 and 86 mg/dl/day, while the plasma TC levels were only 8.9 and 7.5 nmol/ml and NEFA levels were 0.52 and 0.61 meq/l for the respective plasma sample. One infant in Group I exhibited hypertriglyceridemia (TG was 179 mg/dl) while the TC level was 7.1 nmol/ml and the infant had received most of the dietary fat as MCT (Portagen, 88% MCT). The average period of time that intravenous fat was the sole source of fat intake in Group I was 6 days, at which time Portagen feedings were usually begun. This might account for why these infants did not receive a high dose of intravenous fat.

Therefore, most infants in Group I had a relatively small dose of long-chain TG when plasma carnitine was at the lowest levels. This may explain why there was not an extreme elevation of TG in Group I infants during this period. However these infants did not receive a 100 kcal/kg/day energy intake until day 56, and may have benefitted from a higher intake of fat.

The changes of plasma carnitine and triglyceride levels were positively correlated in both Groups II and III (Table 13, p. 68). Infants in Group II, like Group I, were all placed on TPN during hospitalization. All but 2 infants were given simultaneous intravenous lipid emulsion. Once again, during the period of time when the plasma carnitine concentrations were depressed and/or still decreasing (during TPN, fat emulsion, and Portagen feedings), only a

small dose of long-chain TG was given to these infants. The dose of intravenous lipid administered was typically less than 1.0 g/kg/day and the highest isolated dose administered was 1.46 g/kg/day, followed by a dose of 1.35 g/kg/day. The corresponding TG levels were 37 and 79 mg/dl and the TC levels 16.4 and 12.6 nmol/ml. As carnitine-containing feedings began (Enfamil-Premature Formula, Enfamil-20, and breastmilk), there were significant increases of TC and NEC. Although not always significant, there was a trend that TG also increased during this time while accompanied by an increased intake of long-chain TG (Tables 9-11 and Figures A-4-6).

Group III infants also showed a positive correlation between plasma carnitine and triglyceride levels (Table 13, p. 68). While 9 of the 16 infants were placed on TPN, only 3 infants received simultaneous intravenous fat emulsion. The highest isolated fat dose during fat emulsion administration was only 0.83 g/kg/day. Most infants in Group III began receiving carnitine-containing formula which also contained a high percentage of long-chain TG (Enfamil-20 or breastmilk) soon after initiation of TPN. Therefore the levels of TC and NEC increased in Group III infants within the first week of life (Figure 4, p. 46). With the intake of Enfamil-20 and breastmilk, there was also a trend for TG to increase (Tables 10 and 11, pp. 59-60), which would be a reflection of their high content of long-chain TG (Table A-1).

No group showed a significantly negative relationship between plasma carnitine and TG during the study (Table 13, p. 68). However, there was a trend that infants of Group I showed a slight negative

correlation between the two parameters (r=-0.098 and 0.084 for NEC and CNE with TG, respectively). This was in accordance with the observations of DeLeeuw et al. (97) who found that prematurity was one of the most important factors leading to hypertriglyceridemia in newborns. It may be significant that only Group I had a negative correlation between plasma carnitine and TG. This might indicate that these infants had an inadequate reserve of carnitine with subsequent accumulation of TG in the plasma.

Another objective of this study was to observe the changes in plasma carnitines and TG with various nutritional interventions. The interventions included were TPN, fat emulsion, Portagen, Enfamil-Premature Formula, Enfamil-20, and breastmilk. For reference, the carnitine and fat content of each of these are presented in Table 5 (p. 51) and Table A-1.

During the first 24 hours of TPN administration all infants exhibited a decrease TC, NEC, and AIAC. When compared to pre-TPN values, only Groups II and III experienced a significant decrease in plasma NEC and TCE at 24 hours after the initiation of TPN. Infants in Group I had the largest mean decrease in NEC and CNE, but due to a large variance within the group these changes were not statistically significant.

As the duration of TPN feedings advanced, Groups I and II continued to show a decline of plasma carnitines, while only a small depression of carnitine was maintained in Group III. In Group III, the levels of NEC and TC were increased over the 24 hour post-TPN level, but were still below the pre-TPN level. These increases are

attributed to 2 infants of this group who had a small increase of NEC and TC during TPN administration and before they had received dietary carnitine. This finding may indicate that the older infants were able to synthesize carnitine. However, both of these infants began receiving phototherapy treatments for hyperbilirubinemia during 24 hour post-TPN and the discontinuation of TPN. Phototherapy has been shown to result in lipid peroxidation with subsequent red blood cell hemolysis in infants (98). Borum (57) proposed that up to 70% of the blood carnitine is present in the red cells. It is conceivable that the observed increases of carnitine may reflect a medical treatment, rather than infant carnitine synthesis.

The decrease of plasma carnitines in infants on TPN is in accordance with other studies (73,77). This is in contrast to adults, who maintain plasma carnitine levels for at least 20 days during administration of TPN (99). The plasma TG levels remained constant except in Group II where a significant decrease was observed at 24 hour post TPN. There were no significant changes in plasma TG levels compared to pre-TPN levels in any group upon discontinuation of TPN. In case the infants were receiving a carnitine-containing feeding along with TPN, the TG value used in calculations was the one obtained prior to coadministration of the carnitine-containing formula.

The administration of intravenous fat emulsion did not result in a significant change of plasma carnitine in any group (Table 7, p. 54 and Figure A-2). However there were slightly increased levels of NEC and TC in all groups 24 hours after initiation of lipid emulsion even though this preparation does not contain carnitine. This might

reflect a decreased urinary excretion of carnitine, which was observed by Konig et al (100), in adults after administration of intravenous lipid emulsion. Unlike the observations made in the infants of this study, Konig found 16% lower plasma carnitine levels after intravenous lipid emulsion.

The admistration of intravenous fat resulted in a decreased NEC:ASAC ratio in Groups I and III. This was similar to the results in rat studies were provision of a high fat diet caused an increase of acylcarnitine, but not of NEC. This indicated that these infants were probably able to oxidize either the lipid emulsion or endogenous lipid with subsequent production of short-chain acylcarnitines.

There was a decrease in plasma TC and NEC levels during Portagen feedings (Table 8, p. 56 and Figure A-3). This was an expected result since Portagen does not contain a significant amount of carnitine. These decreases were significant in Groups I and II, but not in Group III. Group I had a larger decrease of TC and NEC than Group II. However, there was also a longer time interval between the pre- and post-Portagen values due to the longer period of time needed to attain a full strength formula rate in the Group I infants, (19 days in Group I versus 9 days in Group II). There were no statistically significant changes seen in triglyceride when compared to pre-Portagen values in any group. Group I infants had a decrease in plasma TG despite a higher intake of dietary fat (4.16 g/kg/day), while Group III had a large increase of plasma TG while they received only 1.08 g/kg/day of dietary fat.

Infants placed on Enfamil-Premature Formula showed a substantial increase in the mean TC and NEC levels (Table 9, p. 57 and Figure A-4). However, only Group II had a statistically significant increase in these parameters. In Group I, the increase of NEC and TC levels were not statistically different from pre-Enfamil-PF values due to the small number of infants who received Enfamil-PF. Also, 1 of the 3 infants in Group I showed only a slight increase in TC and NEC (pre: 6.7 nmol/ml and post: 9.3 nmol/ml) whereas the other 2 infants showed a marked increase in both of these parameters (pre: 7.6 and 8.5 nmol/ml; and post: 27.0 and 33.6 nmol/ml) Reasons for this infant not showing a rapid increase in plasma carnitine as did the others may be that there was a decreased gastrointestinal absorption, or an increased renal excretion, or an increased tissue accretion of carnitine during this time.

Plasma TG increased in each group during Enfamil-PF administration (Table 9, p. 57). There was a significant increase of plasma TG only in Group II. These infants also had the highest mean increase of fat intake between pre- and post-Enfamil-PF values. This might explain why only Group II had an increase in TG. Although fat intake was relatively high in Groups I and III (5.23 and 4.71 g/kg/day, respectively), there was no hypertriglyceridemia (TG>160 mg/dl) observed at 24 hours after receiving full strength Enfamil-PF. However, Enfamil-PF contains a relatively high concentration of medium chain triglycerides (43%) which are known to reduce the incidence of hypertriglyceridemia. Enfamil-PF also contains a relatively high calcium concentration. A high calcium intake has been shown to

decrease the fat absorption coefficient (101) which may prevent an elevation of plasma TG in infants on this formula.

During Enfamil-20 feedings, there were significant increases of TC and NEC in all groups (Table 10, p. 59 and Figure A-5). This is reflected in the changes of plasma carnitine with the increased intake of dietary carnitine (Table 12, p. 62 and Figures 7-10, pp. 63-66). Plasma TG increased in all groups, but only significantly in Group III, perhaps because Group III infants had the largest increase in fat intake. This is evident from the high correlation ($r=0.634$) between plasma TG level and fat intake in Group III (Table 15, p. 71).

Breastmilk feedings also resulted in an increase in the levels of all fractions of plasma carnitine and in plasma TG (Table 14, p. 68). Although Warshaw and Curry (102) found that term infants which received breastmilk had higher plasma NEC levels than infants on commercial infant formula, and that this is supposedly due to a higher bioavailability of carnitine in breastmilk, this did not appear to occur in this study of premature infants. However, most infants in this study were not given breastmilk prior to other carnitine-containing formula, which would not allow a true comparison between the rise of plasma carnitine between the two types of feedings.

The results of carnitine determination of infant formula (Table 5, p. 51) confirmed once again that infant formula based on soybean protein are deficient in carnitine and those formula that are milk-based have a high concentration of preformed carnitine (44). However, the carnitine levels of the two milk-based formula were

markedly different from one another. This might be explained by the addition of demineralized whey to Enfamil-Premature formula, which contained almost double the amount of carnitine found in Enfamil-20. Ohtani (43) suggested that formulas with a high whey to casein ratio have a higher concentration of carnitine. However, both Enfamil-PF and Enfamil-20 contain a 60:40 ratio of whey:casein proteins. Therefore, the difference in carnitine concentration between the two formulas may be due to the different protein contents (Enfamil-PF, 2.4 g/dl; Enfamil-20, 1.5 g/dl), or to their concentration, rather than to the addition of whey.

Thus, it appeared that plasma carnitine decreased in all groups receiving a carintine-free diet but increased once dietary carnitine was introduced into the infant diet. The level of plasma carnitine corresponding to a given dietary carnitine intake (μmol/day) was similar between groups. As shown in Table 12 (p. 62), the rate of plasma carnitine increase was highest in Group I (slope=0.596) compared to Groups II and III where the slopes were similar (0.431 and 0.436 respectively). This may be explained by the lower plasma concentrations of carnitine attained in Group I before initiation of a carnitine-containing diet. When carnitine intake was expressed as μmol/kg/day, the increase of plasma carnitine compared with carnitine intake was similar between all groups.

The plasma NEFA levels were determined on 44 samples from infants in Group I. The NEFA level was positively correlated with the plasma level of TC (Figure 11, p. 69). This is not in accordance with most studies (24,75,76). However, these NEFA values included birth NEFA

levels which are known to be elevated due to the endocrine response in the post-partum period. The NEFA were determined from plasma samples obtained during administration of intravenous fat emulsion, Portagen feedings, and in some infants, during theophylline and dopamine therapy. Therefore, the NEFA levels reflect either the relatively small doses of fat emulsion, the administration of a high MCT-containing formula, or a drug therapy, each of which may have influenced the plasma NEFA level. Another factor that must be considered is the length of time the plasma samples were stored before the NEFA levels were determined. Most were kept for several months at -20°. Forbes reported that the serum NEFA concentration may increase up to 63% within 24 hours when stored at -15° (103), while Gordon found plasma NEFA may be stable for several weeks when kept at -20° (104). Since the primary focus of this study was to observe plasma carnitine changes during infancy, the carnitine determination was done in advance of either TG or NEFA to insure an adequate plasma volume for the carnitine assay. The NEFA determinations were made only as a probing observation as a guide for future studies and will not be considered in the final summary of this study.

Based on this study and the research of others, it appears that premature infants have deficient plasma carnitine levels which may indicate a tissue carnitine deficiency as well. Since this population is heavily dependent on dietary carnitine which may not be available to most of these infants, it is necessary to determine (1) the relationship between the plasma and tissue pools of carnitine during infancy, (2) the ability of the premature neonate to utilize NEFA as

evidenced by the plasma ketone and NEFA levels, and as related to the carnitine pools, (3) the optimal intravenous lipid doses needed for the high energy needs of the premature infant without causing lipid intolerance as related to the carnitine pools, and (4) the effect of carnitine supplementation (both oral and intravenous) on the plasma lipid profile on the premature infant.

CHAPTER VI

SUMMARY

This study was designed to determine the plasma carnitine status of premature infants from birth until discharge from hospital care. Plasma carnitine levels were determined in 3 groups (based on birthweight) of premature infants at birth, in maternal and in umbilical cord plasma to assess the relationships between the three at various stages of prematurity. The groups were: Group I <1000 g, Group II 1001-1510 g, Group III 1511-2500 g, and Group IV (reference group) consisted of full term infants. After birth, plasma carnitine levels were determined daily for the first week (except day 6) and then weekly to monitor plasma carnitine changes with advancing age. Plasma carnitine was also determined before and after various nutritional interventions to observe the effects of both carnitine-free and carnitine-containing diets. Carnitine levels were related to plasma lipid levels to determine whether a significant correlation exists between these two variables.

The results showed that plasma carnitine levels are higher in the premature infants with a birthweight <1510 g than in premature infants with a birthweight of 1511-2500 g or full term infants. The level of plasma carnitine in the infant was higher than either maternal or umbilical cord plasma.

After birth, the infants in this study with only two exceptions required an exogenous source of carnitine for maintenance of plasma carnitine. Two infants in the highest birthweight group showed a

slight rise in non-esterified and total carnitine while receiving total parenteral nutrition. Since total parenteral nutrition does not contain preformed carnitine, the increase of plasma carnitine may have reflected endogenous synthesis of carnitine. This increase might have been due to carnitine release from red blood cells, since both of these infants received phototherapy during the time when plasma carnitine increased. This has not been reported in the literature and would warrant further research.

The results furthermore showed that infants given a carnitine-containing formula responded with an increase in plasma carnitine. There was a positive correlation between TC and TG for Groups II and III ($r=0.308$ and $r=0.225$, $p<0.01$). However, there was a small, but insignificant, negative correlation between plasma carnitine and triglyceride in Group I ($r=-0.098$). There were no episodes of hypertriglyceridemia when total carnitine was less than 26 nmol/ml, even though some of the infants had a decrease in carnitine to a level below 10 nmol/ml while on a carnitine-free diet. It must be noted that only small amounts of fat in the form of long-chain fatty acids were given during the period when plasma carnitine levels were the lowest.

Thus it appears that plasma carnitine was dependent upon an exogenous source of carnitine in the premature infant. But a depressed plasma carnitine was not necessarily associated with hyper-triglyceridemia at the level of fat intake provided in this study. Further research is needed to determine if plasma carnitine and tissue carnitine levels are related, and if so, would these also be related

to an elevation of the NEFA:albumin molar ratio and to inability of the infant to produce ketone bodies. If fat intolerance was found to be correlated with a depressed carnitine concentration, there may exist a need for carnitine supplementation in this population.

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APPENDIX

Table A-1. Fat Content of Infant Formulas[1] and Liposyn.[2]

Fat Content	Portagen	Enfamil-PF	Enfamil-20	Liposyn	Breastmilk[3]
Total Fat (g/dl)	3.2	4.1	3.8	10	3.76
Calories as fat (%)	40	44	50	98	55
Source of Fat (%)					
Corn Oil	12	40	0	0	NA
Safflower Oil	0	0	0	100	NA
Soybean Oil	0	0	45	0	NA
Coconut Oil[4]	0	20	55	0	NA
MCT Oil	88	40	0	0	<4

[1] Meade-Johnson product information
[2] Ross-Abbott product information
[3] Reference 105
[4] Coconut Oil contains 59% medium-chain fatty acids (≤C:12) (Ref. 106)

Table A-2. Plasma Carnitine Levels (nmol/ml)[1,2] at Birth[3] for Maternal, Unbilical Cord, and Infant Plasma (Paired).

Group	n	Maternal	Cord	Infant
Total Carnitine				
I	2	22.5 ± 2.5 a,f	32.3 ± 17.6 a,f	26.3 ± 4.7 a,f
II	3	25.1 ± 9.5 a,f	19.5 ± 1.8 a,f	31.4 ± 9.0 a,f
III	8	16.4 ± 1.7 a,g	22.1 ± 2.7 a,fg	24.6 ± 2.6 a,f
IV	10	16.4 ± 1.0 a,g	20.3 ± 1.1 a,f	21.0 ± 1.4 a,f
Non-Esterified Carnitine				
I	2	15.9 ± 3.9 a,f	14.2 ± 13.1 a,f	18.4 ± 3.7 a,f
II	3	20.0 ± 8.2 a,f	13.5 ± 0.4 a,f	23.7 ± 5.1 a,f
III	8	10.7 ± 0.7 a,g	15.8 ± 1.5 a,f	18.9 ± 2.0 a,f
IV	10	12.2 ± 0.8 a,g	15.0 ± 0.7 a,f	15.2 ± 1.1 b,f
Acid-Soluble Acylcarnitine				
I	2	4.8 ± 1.1 a,f	9.3 ± 4.4 a,f	6.8 ± 0.6 a,f
II	3	4.1 ± 1.5 a,f	4.8 ± 1.7 a,f	6.9 ± 3.6 a,f
III	8	4.7 ± 1.2 a,f	5.6 ± 1.1 a,f	5.0 ± 1.0 a,f
IV	10	3.6 ± 0.6 a,f	4.4 ± 1.0 a,f	5.2 ± 0.7 a,f
Acid-Insoluble Acylcarnitine				
I	2	1.8 ± 0.2 a,f	1.4 ± 0.1 a,f	1.1 ± 0.4 a,f
II	3	0.9 ± 0.1 ab,f	1.2 ± 0.3 a,f	0.8 ± 0.2 a,f
III	8	1.0 ± 0.3 ab,f	1.0 ± 0.2 a,f	0.8 ± 0.2 a,f
IV	10	0.8 ± 0.1 b,f	0.8 ± 0.1 a,f	0.7 ± 0.1 a,f

[1] Values are the group means ± SEM

[2] Means in a row or column without a common superscript letter are different ($p<0.05$)

[3] Plasma samples were obtained within 8 hours post-partum

Table A-3. Plasma Triglyceride Levels (mg/dl)[1,2] at Birth[3] for Maternal, Umbilical Cord, and Infant Plasma (Paired).

Group	n	Maternal	Cord	Infant
I	2	89 ± 7 [a,f]	63 ± 39 [a,f]	39 ± 19 [a,f]
II	3	192 ± 54 [a,f]	34 ± 13 [a,f]	34 ± 8 [a,f]
III	8	166 ± 25 [a,g]	23 ± 4 [a,f]	24 ± 4 [a,f]
IV	10	175 ± 27 [a,g]	30 ± 5 [a,f]	26 ± 15 [a,f]

[1] Values are the group means ± SEM
[2] Means in a row or column without a common superscript letter are different ($p<0.05$)
[3] Plasma samples were obtained within 8 hours post-partum

Table A-4. Ratio of Total Carnitine Levels[1] for Maternal, Umbilical Cord, and Infant Plasma.

Group	Maternal	Cord	Infant
I	1	0.97	1.45
II	1	0.81	1.20
III	1	1.38	1.61
IV	1	1.23	1.28

[1] Values represent the ratio of the mean unpaired total carnitine (TC) levels for each group.

Table A-5. The NEC:ASAC Ratio Changes with Nutritional Intervention.

NEC:ASAC (of group $\bar{x}$)	Group I	II	III
Pre-TPN	2.39	3.30	2.82
24°Post-TPN	2.50	2.63	3.73
Post-TPN	1.79	2.44	3.77
Pre-IV Fat	2.90	2.53	4.58
Post-IV Fat	2.57	5.07	3.65
Pre-Portagen	3.78	4.95	5.96
Post-Portagen	1.88	2.46	3.20
Pre-Enfamil-PF	6.02	1.83	NA
Post-Enfamil-PF	3.85	4.09	NA
Pre-Enfamil-20	1.51	2.06	3.02
Post-Enfamil-20	4.67	3.61	2.28
Pre-Breastmilk	NA	3.79	3.14
Post-Breastmilk	NA	3.69	3.06

Table A-6. Correlation Between Plasma Triglyceride Concentration and the NEC:ASAC Ratio.

Group	r	p
I	-0.011	0.19
II	-0.131	0.12
III	-0.149	0.062

Variables defined in text

Table A-7. Correlation Between Plasma Triglyceride and Acid-Insoluble Acylcarnitine Concentrations.

Group	r	p
I	0.004	NA
II	-0.101	NA
III	-0.127	NA

Variables defined in text

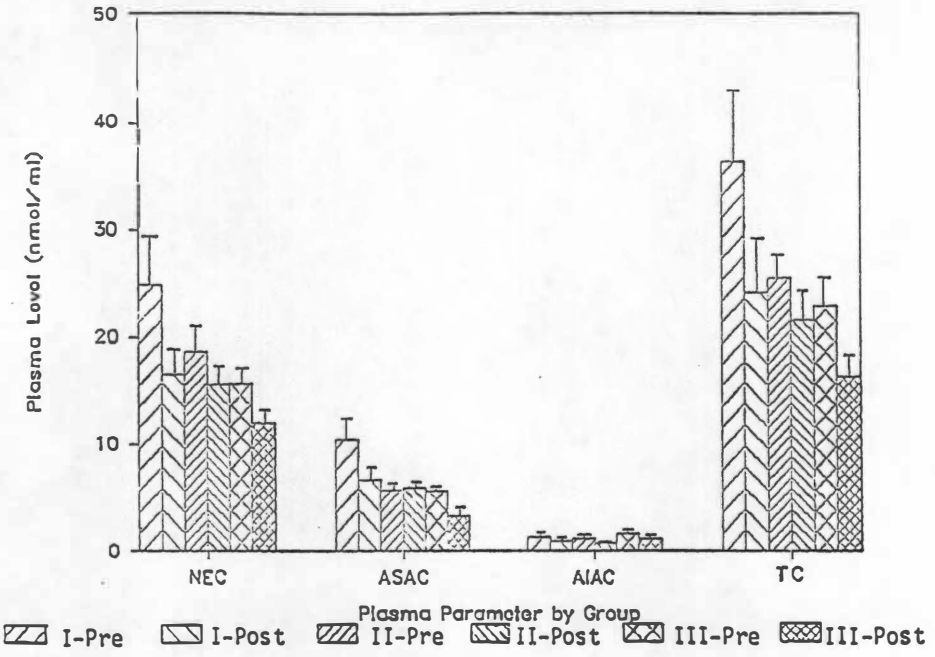

Figure A-1. Carnitine Fractions by Group Before and 24 Hours After Initiation of TPN.

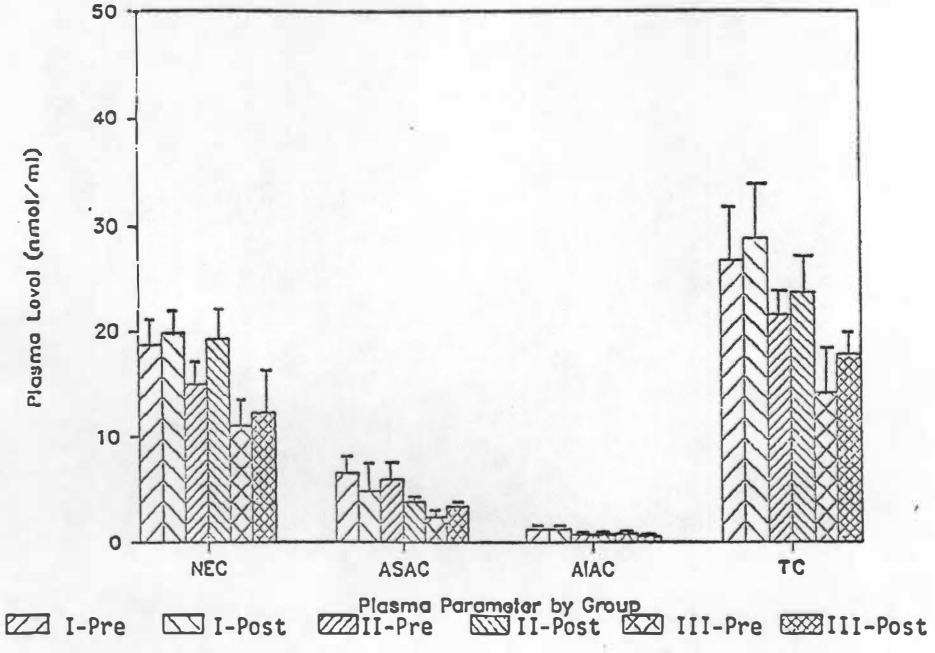

Figure A-2. Carnitine Fractions by Group Before and 24 Hours After Intravenous Lipid Infusion.

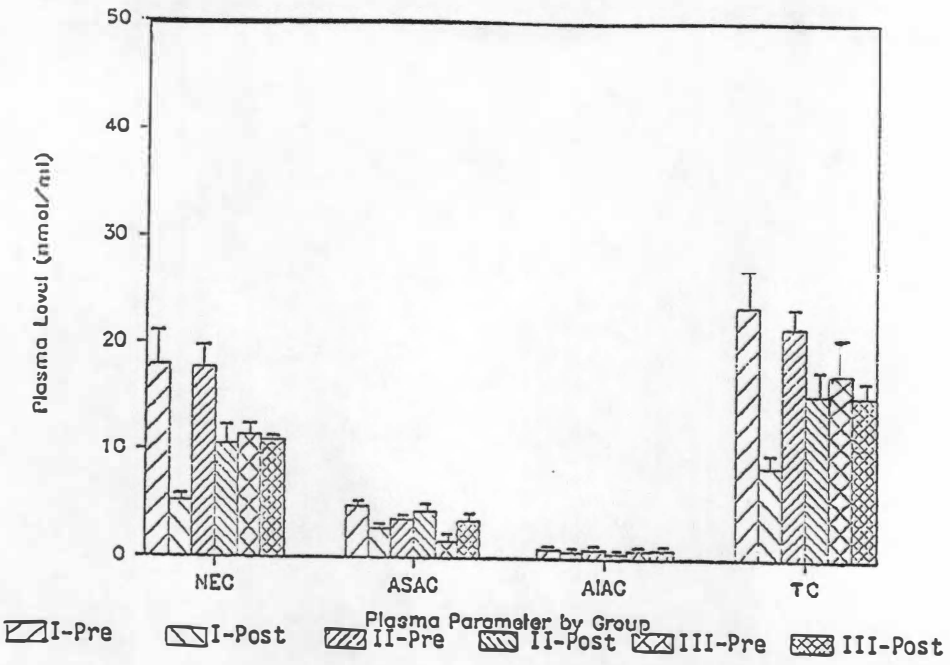

Figure A-3. Carnitine Fractions by Group Before and 24 Hours After Achievement of Full Strength Portagen.

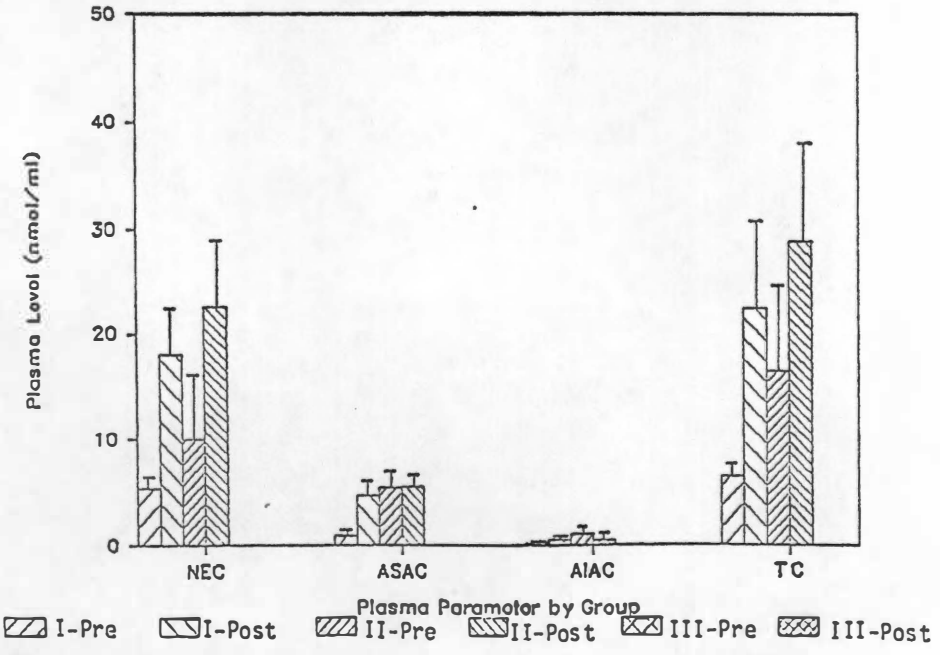

Figure A-4. Carnitine Fractions by Group Before and 24 Hours After Achievement of Full Strength Enfamil-Premature Formula.

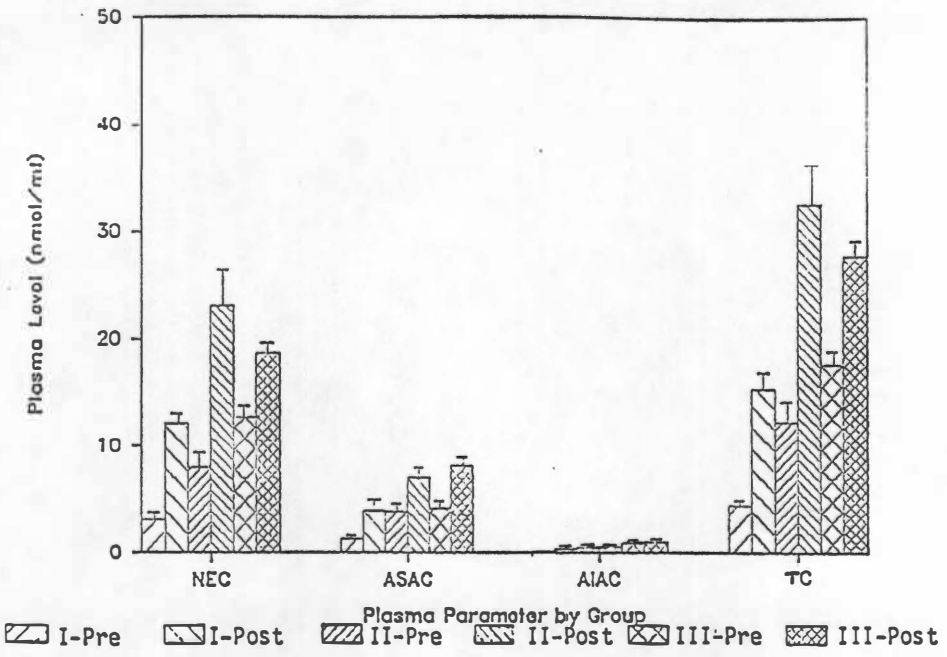

Figure A-5. Carnitine Fractions by Groups Before and 24 Hours After Achievement of Full Strength Enfamil-20.

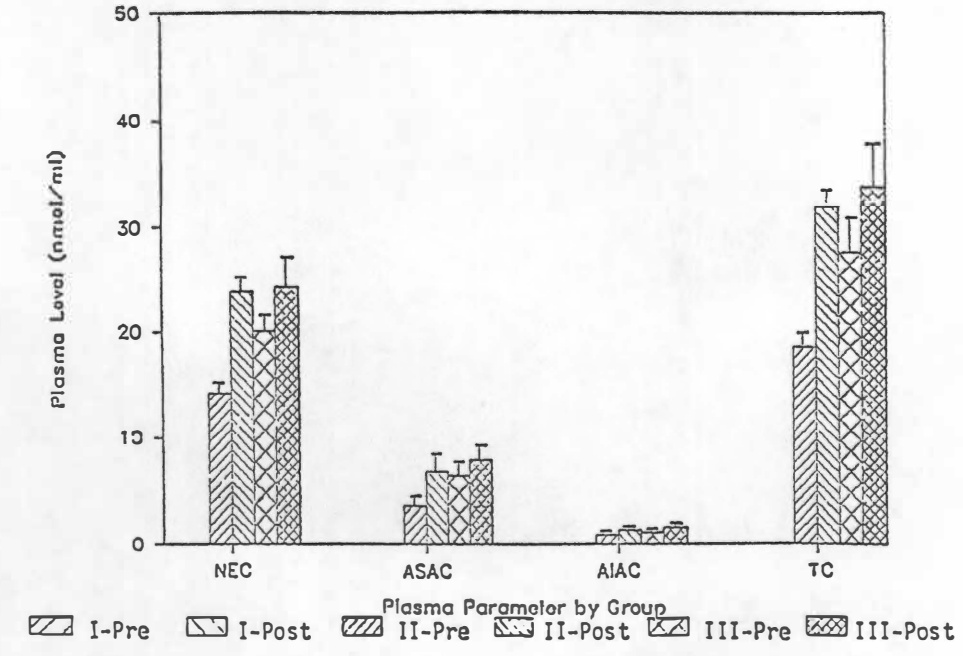

Figure A-6. Carnitine Fractions by Group Before and 24 Hours After Achievement of Full Strength Breastmilk.

VITA

Rebecca Smith was born in Sherman, Texas, on May 21, 1958. She attended Wakefield Elementary and Piner Junior High Schools and graduated from Sherman High School in Sherman, Texas, in May of 1976. She attended The University of Texas at Austin and graduated from The University of Texas Health Science Center at Dallas, in 1980 with a Bachelors of Science in Nutrition. She worked at Parkland Memorial Hospital for three years as a Clinical Dietitian with a specialty in thermal injury and neurosurgical patient care. Her work toward a Master's degree in nutrition science was begun in July of 1983. During this time, she worked as a laboratory research assistant for the Department of Nutrition and Food Science. After receiving the Master's degree in December of 1985, she plans to pursue research endeavors in nutrition as well as other areas, with possible plans of pursing another graduate degree.